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CAROTIN

THE PRINCIPAL NATURAL YELLOW PIGMENT OF MILK FAT

CHEMICAL AND PHYSIOLOGICAL RELATIONS OF PIGMENTS OF MILK FAT TO THE CAROTIN AND XANTHOPHYLLS OF GREEN PLANTS

BY

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SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

IN THE

GRADUATE SCHOOL

OF THE

UNIVERSITY OF MISSOURI

1913

Chem 1109.13.11

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CAROTIN—THE PRINCIPAL NATURAL YELLOW PIGMENT OF MILK FAT.

Its Relations to Plant Carotin and the Carotin of the Blood Serum, Body Fat and Corpus Luteum.

LEROY S. PALMER AND C. H. ECKLES.

The investigations dealing with the natural yellow pigment of milk fat will appear as a series of four bulletins as follows:

Part. I. A Review of the Literature Concerning the Yellow Plant and Animal Pigments. Missouri Agricultural Experiment Station Research Bulletin No. 9.

Part II. The Chemical and Physiological Relation of the Pigments of Milk Fat to the Carotin and Xanthophylls of Green Plants. Missouri Agricultural Experiment Station Research Bulletin No. 10.

Part. III. The Pigments of the Body Fat, Corpus Luteum and Skin Secretions of the Cow. Missouri Agricultural Experiment Station Research Bulletin No. 11.

Part IV. (A). The Yellow Lipochrome of Blood Serum. (B.) The Fate of Plant Carotin and Xanthophylls During Digestion. (C). The Pigments of Human Milk Fat. Missouri Agricultural Experiment Station Research Bulletin No. 12.

The present paper is the first of the series. As indicated it will be confined entirely to a review of the extensive literature in regard to the yellow plant and animal pigments.

Part II will be a report of the chemical identification of the milk fat pigment. It will also include a number of investigations showing the relation between the amount of pigment in the milk fat and the character of the ration and the breed of the cow.

Part III will consist of the data showing the chemical identification of the pigments mentioned. Data will also be presented showing the relation between the color of the body fat and the character of the

ration and the breed of the cow. A brief experiment will also be reported showing the absence of these pigments in the body of a newborn Jersey calf.

Part IV (A) will report the chemical identification of the blood serum pigment. It will show how blood carries the pigment and what effect the character of the ration has upon the amount of pigment carried by the blood and the amount secreted in the milk at the same time. A brief study of the cause of the high color of colostrum milk will also be reported. (B) This will consist of the report of a few investigations relative to the fate of the carotin and xanthophylls of plants during their passage through the cow's body. (C) The experiments reported here will show the character of the pigments of human milk fat.

A REVIEW OF THE LITERATURE CONCERNING THE YELLOW PLANT AND ANIMAL PIGMENTS.

It has been the custom for generations to judge the quality of dairy products to a large extent by their yellow color. This has been carried to such an extent that the manufacturer of butter, whether it be on a large or small scale, finds it impossible to market butter that does not have a standard yellow color. The consumer of milk or cream as a rule looks upon a yellow color as indicating the richness and quality of the product. Although it is well known that the color has no relation to the food value of milk or cream, the popular prejudice is so strong that the producer of market milk has to take it into account and try to supply a product with as much natural yellow color as possible.

During part of the year, namely during the spring and early summer and usually also in the early fall, the fresh green feeds which the cows receive give the shade of yellow to the milk fat which the consumer demands. During the winter months, or in summer if the pastures become dry, this yellow color is wholly or in part absent from the milk fat, and the butter manufacturer is then forced to color the butter artificially, in order to maintain the required standard.

It is generally accepted as a fact that the breed of the cow has a pronounced relation to the color of the milk fat and that the Guernsey and Jersey breeds rank first in this respect. The breeders of this class of cattle have emphasized this characteristic as one of the strong points of their respective breeds. This characteristic of Guernsey and Jersey breeds, as compared with the Holstein and Ayrshire breeds, has been generally attributed to physiological differences. According to this view, Guernsey and Jersey cattle are able to produce a higher colored fat due to some inherent quality, just as they are able to produce a higher percentage of fat in their milk. It is a well-known fact that the skin and the secretions of the skin of Guernsey and Jersey cattle have a higher yellow color than other breeds, and this characteristic is looked upon by cattle breeders as an indication of the ability of animals of these breeds to produce highly colored milk fat.

The body fat of Guernsey and Jersey cattle is also characterized by a high yellow color and for this reason beef from these animals is often looked upon with disfavor by the butcher and the consumer.

That the yellow color of butter has a relation to its market value is shown by the fact that "color" has a place on the standard butter score cards with a value of fifteen out of one hundred points. The

oleomargarin manufacturers have also recognized the value of color and, so far as the law has permitted, have made a practice of coloring oleomargarin in imitation of butter. When the law placed a tax on artificially colored oleomargarin, or in some cases prohibited it entirely, the manufacturers began using only the highest colored beef fats that could be bought or mixed the oleomargarin with butter having a high natural color, in order to produce the color they sought.

The Pigment of the Butter Fat as a Factor in the Coloration of Milk.

The more or less yellow color of cows' milk which is especially evident in the cream and butter has not been attributed in all cases to the same pigment. On the one hand a few authors have stated that the pigment of butter is manifested in the familiar yellow color of milk whey. This view originated with Blyth [1] who called the whey pigment lactochrome and the view has found its way into a number of texts. On the other hand a larger number of authors have ignored the whey pigment and considered the lipochrome-like pigment of the milk fat to be the only factor causing the yellow color of cream and butter.

The investigations which were carried on in this laboratory have been the first to point out that the whey pigment and the butter fat pigment are not identical but are distinct substances; and that both are of importance in causing the yellow color of milk. The pigment of the butterfat is the more important of the two, however. The pigment of the whey is of secondary importance, and is of an entirely different nature. Its probable identity with urochrome, the specific urinary pigment, has recently been shown by one of us.[2]

Object of the Present Investigations.

The present investigations were undertaken primarily to study the chemical nature of the yellow butterfat pigment and to classify it from a scientific standpoint. At the same time information was gathered with the hope of ascertaining to what extent the generally accepted views concerning the color of milk fat are correct in order to establish a scientific basis for the subject which would be of value to those interested in the handling of dairy products in a commercial way.

In the principal part of the investigation it was sought; (1) to show the chemical and if possible the physiological relation of the butter fat pigment to similar animal pigments such as the

1. A. W. Blyth, "Foods. Their Composition and Analysis" Text, 4th Edition 1896, p. 239.
2. Lactochrome: The Yellow Pigment of Milk Whey, etc., by Leroy S. Palmer and Leslie H. Cooledge. Missouri Agricultural Experiment Station Research Bulletin No. 13;; Jour. Biol. Chem. XVII, p. 251 (1914).

corpus luteum pigment, the body fat pigment, and the blood serum pigment; and (2) to show the chemical and physiological relation of the butterfat pigment to the carotin and xanthophylls of green plants.

In the secondary part of the investigation it was sought to study the influence of certain factors which have both practical and scientific bearing upon the color of the butterfat, among which are the breed of the animal and the character of the ration, the latter in connection with the chemical and physiological studies indicated above.

THE PIGMENTS OF PLANT ORIGIN.

The earliest researches on plant pigments dealt with the green pigments. Caventon first called them chlorophyll in 1817. His work, however, was preceded by the pioneers in this field, among which the names of Grew, whose work is dated 1682, and Rouelle, Meyer, Fourcroy, Berthellot, Senebier, Proust and Vanquelin are of historical interest.

The Carotins.

The Pigment of the Carrot. The yellow pigment of the cultivated carrot (Daucus Carota) has long been of interest to botanists and chemists, the investigations of this body having extended over almost one hundred years.

Wachenroder [1] was the first investigator of the carrot pigment. He isolated it and called it Karotin. The work of Vanquelin and Bouchardat [2] soon followed and a little later Zeise [3] took up the study. He obtained the first crystals and assigned to them the chemical formula $C_5 H_{10}$ or 10 ($C_5 H_8$).

Husemann [4] was the next investigator. He found six per cent of oxygen in his pure preparation and gave the pigment the formula $C_{18} H_{24} O$. A secondary pigment which he thought always accompanied the carotin in small amounts, he named hydrocarotin and gave it the formula $C_{18} H_{30} O$.

It is to Arnaud [5] however that we are indebted for the first thorough research in regard to the carrot pigment carotin. The crystals which he obtained were flat, rhombic-shaped crystals, red orange by transmitted light, and greenish blue by reflected light. They melted at 168° C. He showed beyond a doubt that the pigment was simply

1. Dissertatio de Anthelminticis Göttingen 1826; also Geigers Magaz. Pharm. 33 p. 144 (1831); also Berzelius Jahresber. 12 p. 277 (1833).
2. Schweizg. Jour. Chem. 58, p. 95 (1830).
3. Lieb. Ann. 62 p. 380 (1847); Annal. Chem. Phys. (3) 20, p. 125 (1847).
4. Lieb. Annal. 117 p. 200 (1860).
5. Compt. Rend. 102 p. 1119 (1886), p. 1319 (1887); Jour. Pharm. Chim. 14 p. 149 (1886).

an unsaturated hydrocarbon. He gave it the formula C_{26} H_{38} and the iodine derivative the formula C_{26} H_{38} I_2.

Eüler and Nordenson [1] report the most recent investigations in regard to the carrot pigment. They found their crystalline preparation to be mixed with crystals of xanthophyll; they also showed that the belief often advanced that carotin is chemically related to cholesterol, is unfounded.

The Carotin of Green Plants.—Arnaud [2] was one of the first investigators to show that the carrot carotin is identical in properties with a yellow constituent of chlorophyll, although the existence of this yellow constituent of chlorophyll had long been the subject of investigation.

Berzelius [3] first sought to isolate a yellow pigment from autumn leaves by extracting with alcohol. He called it "Blattgelb" or xanthophyll, and expressed the belief that the pigment pre-existed along with the green coloring matter of the leaf.

The subject subsequently received the attention of many investigators. Fremy,[4] Michels, Millardet, Müller, Tinisnsseff, Gerland, Rannenhoff, Askennasy, Stokes, Sorby,[5] Tschirch,[6] Kraus,[7] Filhol,[8] Hansen,[9] Conrad,[10] Wiesner,[11] and many others took up the investigation.

Fremy designated the yellow pigment Phylloxanthin. Filhol noticed that by treating crude alcoholic chlorophyll solutions with animal charcoal it was possible to remove the green constituent of the mixture leaving a yellow colored solution, the color of which he believed was due to a pre-existing pigment or pigments associated with the green one. Kraus confirmed the observations of Filhol, and was the first to notice that when an alcoholic solution of chlorophyll is shaken with benzoline (petroleum ether) the alcohol retains the yellow coloring matter, the benzoline taking up the green constituent. Kraus' investigation was also the first to show that the ordinary chlorophyll spectrum was due partly to the green and partly to the yellow constituent, which he called xanthophyll. Kraus' xanthophyll gave a

1. Zeit. f. Physiol. Chem. 56, p. 223 (1908).
2. Compt. Rend. 100, p. 751 (1885); 104 p. 1293 (1887).
3. Ann. d. Chem. 21, p. 257 (1837).
4. Ann. Sc. Nat. 13, p. 45 (1860); Compt. Rend. 41, p. 189 (1865).
5. Proc. Roy. Soc. 21, p. 456 (1875).
6. Botan. Zeitung. 42, p. 817 (1884).
7. Flora, p. 155 (1875).
8. Compt. Rend. 39, pp. 9-184; 50, pp. 545 and 1182.
9. Sitz. ber. d. phys. Med. Ges. Würzberg (1883); and Arbeiten d. Botan. Gessel. Würzberg, 3, p. 127 (1884) and "Die Farbstoff des Chlorphylls" (1889).
10. Flora, Vol. 25 (1872).
11. Flora, Vol. (1874); Sitz. der. Wein. Akad. 89, 1. abts. p. 325.

dark blue coloration with concentrated H_2SO_4, and bleached very quickly in the sunlight.

Sorby, using carbon bisulphide as the separator in place of benzoline, was the first to show that there is more than one yellow pigment associated with chlorophyll.

Hansen's method of isolating the yellow pigments was still different. He treated the alcoholic extracts with caustic alkali, evaporated the liquor to dryness and extracted the yellow pigment from the residue with ether, the spectroscopic study of which led him to believe that it exhibited three absorption bands. He believed also that it was identical with the pigment of the carrot.

E. Schunck [1] obtained from all crude alcoholic chlorophyll extracts minute sparkling red crystals which deposited on standing, and which he considered identical with the crystals which Bougarel [2] had called erythrophyll, and which Hartsen [3] has called crysophyll. This pigment showed two absorption bands.

Tschirch [4] using Hansen's method, found two yellow coloring matters, to which he gave the name xantho-carotin, showing three bands, and xanthophyll proper which showed no bands.

Returning now to Arnaud's [5] work, we find that he identified the red orange crystalline pigment which he obtained from spinach leaves with the carotin of the carrot, both as regards to crystalline form, melting point and chlorine derivatives.

We are indebted to Immendorff [6] for the confirmation of Arnaud's results indicating that the carotin of green plants is identical with the carotin of the carrot. Immendorff gave the pigment the formula which Arnaud found for carotin, namely $C_{26}H_{38}$. He states, however, that the percentage composition of the pure pigment corresponded best with Zeise's formula, C_5H_8. Immendorff believed that carotin was the only yellow pigment accompanying chlorophyll in the green leaf.

One of the most extensive publications in regard to carotin is that by F. G. Kohl [7] This author also gives one of the best and most voluminous compilations of the carotin literature that is to be found, besides a large amount of experimental data. The literature is also excellently reviewed by Tammes.[8] Kohl gave carotin the formula

1. Proc. Roy. Soc. 44, p. 449.
2. Ber. Chem. Gessel, 10, p. 1173 (1877).
3. Arch. Pharm. 207, p. 166 (1875).
4. Botan. Zeitung. 42, p. 817 (1884); Ber. der Deutsch. Botan. Ges. 14, pt. 2, p. 76 (1896).
5. Compt. Rend. 100, p. 751 (1885).
6. Landwirtschaftliche Jahrbücher 18, p. 507 (1889).
7. Untersuch, Uber d. Karotin, Leipzig. 1902.
8. Flora, p. 205 (1900).

$C_{26}H_{38}$, and the iodine derivative $C_{26}H_{38}I_2$. He also gave a detailed description of the spectroscopic absorption of carotin. In ether and carbon-bisulphide he measured three bands:

In ether	*In carbon bisulphide*
I 490-475 λ	I 510-485 λ
II 455-445 λ	II 470-458 λ
III 430-418 λ	III 437-425 λ

Carotin is lævorotatory, according to Kohl, α_D at 15° in chloroform being—30.17°.

Schunck [1] in his spectroscopic study of the yellow pigments of leaves and flowers, described the properties of carotin. Schunck also photographed the absorption bands of crysophyll (carotin) from the daffodil leaf, from spinach, from the carrot and from grass, in alcoholic solution. All of the carotin preparations showed the same three pronounced bands situated between F and H the first band of which lay almost directly upon the F line.

The most recent detailed investigation of the carotin of green plants is that of Willstätter [2] and Meig, and a study of their data shows that their results are to be accepted as the final proof of the chemical constitution and properties of this pigment.

Willstätter and Meig describe the properties of carotin as follows: Its crystals are copper colored plates of almost quadratic form, and melt at 167.5° to 168° C. Its crystals are soluble with great difficulty in hot ethyl alcohol and almost insoluble in cold ethyl alcohol, and in methyl alcohol they are still less soluble; one gram of the crystals requires 1.5 liters of petroleum ether (b. p. 30-50 °C.) for solution and about 900 c.cm. of hot ethyl ether; the crystals are difficultly soluble in acetone, easily soluble in benzol, very easily soluble in chloroform and instantly soluble in carbon bisulphide; the crystals are soluble in concentrated sulphuric acid with an indigo blue color and are precipitated as green flakes on dilution with water.

The carotin obtained by Willstätter and Meig crystallized from its deep red carbon bisulphide solution on addition of absolute alcohol, but analysis showed that the crystals contained ½ to ⅔ of a molecule of alcohol of crystallization. The carotin showed the composition of a pure hydrocarbon only after crystallization from low boiling point petroleum ether. From this solvent the preparation of Willstätter and Meig showed the composition C_5H_7. A preparation of carotin which the same authors obtained from the carrot showed the same

1. Proc. Roy. Soc. 72, p. 170 (1903).
2. Ann. der Chemie, 355, p. 1 (1907).

composition. A molecular weight determination of both the carotin from the carrot and from the Brennessel leaves, by the ebullioscopic method in carbon bisulphide gave an average of 533 which corresponds exactly with $8(C_5H_7)$ or $C_{40}H_{56}$. This shows that Arnaud's formula of $C_{26}H_{38}$ is not quite correct. The same difference is brought out by the analysis of the iodine derivative which Willstätter and Meig also prepared.

The absorption bands of carotin were measured by Willstätter and Meig and they coincided almost exactly with those given for it by Tschirch [1] and Monteverde [2]. They did not attempt to measure the third band in the violet which they considered to be end absorption, but measured only the two bands in the blue and indigo blue.

Willstätter and Meig (alcohol sol.)	Tschirch (alcohol sol.)	Monteverde (petroleum ether sol.)
I 488-470 λ	I 487-470 λ	I 491-472 λ
II 456-438 λ	II 457-439 λ	II 461-444 λ

Carotin in Flowers, Fruits and Seeds. According to Czapek [3] carotins have been identified in many flowers by Hansen,[4] Immendorff,[5] Kohl,[6] Tammes,[7] Hilger,[8] and his pupils, Wirth,[9] Pabst,[10] Kirchner,[11] Ehrung [12] and Schuler.[13]

Among the fruits, Arnaud,[14] Passerini,[15] Kohl,[16] Schunck [17] and Montanari [18] have investigated the tomato pigment and believed it to be a carotin. Its identity as a truly isomeric carotin has recently been proved by Willstätter and Escher.[19] Schrötter [20] has shown that the pigment of the pumpkin is in all probability a carotin and Desmoliere [21] has identified carotin in the apricot.

1. Ber. d. deut. botan. Ges. 14, 76 (1896); 22, 414 (1904).
2. Acta Horti. Petropolitani XIII Nr. 9, 123 and 150 (1893).
3. Bichemie der Pflanzen, vol. I, p. 172, etc.
4, 5, 6, 7. Loc. cit.
8. Botan. Centr. 57, p. 335 (1894).
9. Dissert. Erlangen (1891).
10. Arch. Pharm. 230, p. 108 (1892).
11. Dissert. Erlangen 1892.
12. Botan. Cent. 69, p. 154 (1897).
13. Dissert. Erlangen. 1899.
14. Compt. Rend. 102, p. 1119 (1886).
15. Compt. Rend. 100, p. 875 (1885).
16. Loc. Cit.
17. Proc. Roy. Soc. 72, p. 172 (1903).
18. Le Staz. sp. agra. ital. 37, p. 909 (1904).
19. Zeit. Physiol. Chem. 64, p 74 (1910).
20. Vehr. Zool. bot. Gessel. 44, 298 (1895).
21. Chem. Centr. 2, p. 1001, 1902.

Among the seeds, Schunck [1] has found the annatto pigment to be a carotin.

The Xanthophylls.

It was mentioned above that it has been found that a second class of pigments usually accompanies carotin. Investigations of this class of pigments, now called xanthophylls, has not been as extended as that of carotin but the constitution and properties of the xanthophylls are nevertheless at present established.

Sorby [2] differentiated the pigments accompanying chlorophyll as xanthophyll, orange xanthophyll, and yellow xanthophyll, all with spectroscopic properties. J. Borodin [3] observed that besides carotin, a second crystallizable yellow substance exists in leaves which is much more soluble in alcohol than carotin and insoluble in benzine. Immendorff [4] denied the existence of more than one pigment as was noted above. Monteverde [5] confirmed Borodin's observations. Tschirch [6] in 1896, showed that green leaves contain a second yellow pigment which, however, showed no absorption bands. Tschirch called the second pigment xanthophyll. The name, however, was a misnomer, for Schunck [7] later showed that Tschirch was dealing with a group of water and alcohol soluble pigments probably identical with the lichnoxanthine described by Sorby.[8] Tschirch [9] later recognized the existence of a true second yellow crystallizable pigment.

Molisch [10] in his critical study of the yellow pigments left the question of their plurality an open one, and Tammes [11] also left the question undecided.

Schunck [12] in his widely known spectroscopic study of the yellow pigments of plants and flowers, demonstrated beyond a doubt that a second great group of pigments, which he designates the xanthophylls, accompanies the crysophyll. He differentiated three different xanthophylls and designated them L. B. and Y. xanthophyll, respectively.

He found that the xanthophylls were all characterized by giving the same color reactions in the dry state as crysophyll and three

1. Proc. Roy. Soc. 72, 1903.
2. Proc. Roy. Soc. 21, p. 457 (1875).
3. Melanges Biol. tir. d. bull d. L'Acad. Imp. d. St. Petersb. 11, p. 512 (1883).
4. Loc. cit.
5. Loc. cit. p. 148 (1903).
6. Ber. d. d. Botan. Gessel, 14, p. 76 (1896).
7. Proc. Roy. Soc. 72 (1903).
8. Loc. cit.
9. Ber. d. d. Botan. Gessel. 22, p. 414 (1904).
10. "Die Krystallization und der Nachweis des Xanthophylls (carotins) in Blatte" (Ber. d. Deut. Botan. Ges. 14, p. 18 (1896).
11. Loc. cit. (1900).
12. Proc. Roy. Soc. 65 (1899); 68 (1901); 72 (1903.)

similar absorption bands in the violet region of the spectrum. The bands of the xanthophylls, however, were all shifted somewhat towards the blue with respect to the bands of crysophyll, the amount of shifting depending on the xanthophyll, L xanthophyll being shifted the least and Y xanthophyll the most. Schunck found the absorption bands of the different xanthophylls especially characterized by the action of their alcoholic solutions in the presence of HCl and HNO_3 the details of which are given in his latest paper.[1]

Schunck also made the very interesting discovery that the yellow pigment of egg yolk and fowl serum shows the identical properties of L xanthophyll both with respect to the position of the original absorption spectra and also the action of acids upon the spectra.

One of the most interesting and important studies of chlorophyll and its accompanying yellow pigments was made by Tswett[2] who discovered and thoroughly investigated the adsorption properties of these pigments. He was able to demonstrate the presence of at least four different xanthophylls which he designates as xanthophylls α α' α'' and B. A more detailed review of this work will be given in connection with a report of the present investigations. It is of interest here especially on account of its historical position with respect to the establishment of the chemical constitution of the xanthophylls.

It was Willstätter and Meig[3] who isolated and identified the crystalline xanthophyll pigment accompanying the carotin in green plants and leaves, and, as noted above, Eüler and Nordenson[4] have recently found xanthophyll crystals in their extracts from the carrot, thus indicating a more general distribution of the xanthophylls in connection with carotin than has been believed.

The results of the study of the crystalline xanthophyll show that it is composed of carbon, hydrogen and oxygen in the proportion C_{40} H_{56} O_2 and is thus merely carotin dioxide.[5] The pigment is further distinguished from carotin by the color and shape of its crystals, which are yellow or orange trapesium plates sometimes spear or wedge-shaped which are characterized by a steel blue reflection. The pigment exhibits an entirely different solubility toward petroleum ether and absolute alcohol than carotin, being insoluble in the former and readily soluble in the latter solvent. According to these authors, the pure

1. Proc. Roy. Soc. 72 (1903).
2. Ber. Botan. Gessel, 24, pp. 316 and 384 (1906).
3. Ann. der. Chemie, 355, p. 1 (1907).
4. Loc. cit.
5. Willstätter and Meig point out the probable identity of xanthophyll with the hitherto unexplained hydrocarotin found by Husemann.

crystals have a melting point of 172° C. (corrected) which is slightly higher than the melting point of the carotin crystals; and the absorption bands of the pigment are slightly shifted toward the violet from the corresponding bands of carotin, as was also shown by Schunck [1] for the xanthophylls which he differentiated.

It might be readily assumed that xanthophyll is formed directly from carotin in the plant. In fact Tschirch [2] has claimed that carrot carotin goes over to xanthophyll in the air. Eüler and Nordenson [3] do not credit this statement and state that, "One may well suppose that in the plant, xanthophyll normally is formed from the carotin, but outside of the plant it has not been possible to imitate this transformation, the most skillful oxidation always leading to a much higher oxidized product." Willstätter and Meig believed in this connection that xanthophyll although carotin dioxide is not the end product of the oxygen absorption of carotin in the plant. Monteverde [4] and Lyubimenko have recently claimed that chlorophyll and xanthophyll originate from the same colorless substance, carotin being a complimentary product generated during the formation of cholorophyll, but not necessarily from the xanthophyll.

The Pigments of Animal Origin.

The Luteins.—We will now direct our attention to a review of the literature bearing upon the yellow pigments of so-called animal origin. Thudichum [5] was one of the first to investigate the yellow animal pigments and he classified a great many of them together with the yellow pigments of plants under the name lutein, the name being taken from the pigment of the corpus luteum. He states, "Various parts of animals and plants contain a yellow crystallizable substance which has hitherto not been defined, and which I call lutein. It occurs in the corpora lutea of the ovaries of animals, the serum of the blood, the cells of adipose tissue, in butter, in the yolks of eggs of oviparous animals, in seeds such as maize, in husks and pulps of fruits such as annatto, in roots such as carrots, in leaves such as those of coleus, and in the stamens and petals of a great many flowers."

It is unfortunate that none of the above statements are supported by experimental evidence, for it can hardly be accepted that Thudichum was able to obtain crystals of lutein from all the bodies in which he

1. Loc. cit.
2. Ber. Botan. Gessel. 22, p. 414 (1904).
3. Loc. cit.
4. Bull. Acad. Imper. Sc. St. Petersb. 30, p. 609 (1912).
5. Proc. Roy. Soc. 17, p. 253 (1869).

claims to have found it, or was able to show all the properties which he describes for the crystals which he evidently did obtain.

Crystalline animal pigments were apparently obtained before Thudichum's claims in this regard. According to Krukenberg,[1] Wittich[2] obtained crystals of a red pigment from Euglenia Sanguirubo, and Piccolo and Lieben[3] found a crystalline animal pigment. Pouchet[4] a little later obtained a yellow crystalline pigment from lobsters.

The Chromophanes.—The early workers in the field of animal pigments laid great emphasis upon the so-called color reactions, one of which, the blue reaction which concentrated HNO_3, was mentioned by Thudichum. That a similar reaction is given by concentrated H_2SO_4 was first noticed by Wittich in 1863, and Buchholz also noticed it with a fat pigment from a Ganglion cell of an invertebrate. Piccolo and Lieben had also noticed the blue reaction with concentrated H_2SO_4. Besides Thudichum, Filhol[5] and Städeler[6] noticed the blue reaction with concentrated HNO_3. Städeler attempted to isolate the egg yolk pigment. He failed to do so, however, but attempted to establish the difference between it and Bilirubin with which it had been considered identical. A little later a third reaction of the luteins was discovered by Schwalbe,[7] namely a blue-green color with a solution of iodine in potassium iodide. Schwalbe first noticed the reaction with the cone-globules of the retinas of birds and lizard's eyes. The red globules gave a beautiful blue to blue-black color, and the yellow oil globules a green to blue-green to blue. The pigments thus characterized were called chromophanes by Schwalbe and the existence of these pigments was a little later considerably extended by Capranica[8] who also made use of the iodine reaction.

Kühne[9] took up the study of the chromophanes of the cone-globules of bird retinas, and separated three pigments which he designated Rhodophan, Chlorophan and Xanthophan, respectively, according to the color of their solutions.

Kühne also studied the absorption spectra and color reactions of the pigment of the egg yolk and the corpus luteum and compared them

1. Grundzuge einer vergleichenden Physiologie der Farbstoff und der Farben; 1884.
2. Arch f. Path. Anat. 27, p. 573 (1863).
3. Giornals d. Scienze Naturali et. Economich. Palermo 2, p. 258 (1866).
4. Jour. d. L'Anat. et. Physiol. 12, p. 12 (1876).
5. Compt. Rend. T. 39, p. 184, T. 50, pp. 545 and 1182.
6. Jour. f. Pract. Chem. 100, p. 149 (1867).
7. Hand D. Ges. Augenheilkunde von Graefe u. Saemisch I, p. 414 (1874).
8. Arch. f. Anat. Physiol. p. 283 (1877).
9. Untersuch, des Physiol. Universität Heidelberg I, 4th Heft, p. 341 (1878); IV, p. 169 (1882); Jour. Physiol. 1, p. 109 (1878).

with these properties of the retinal pigments. A study of their spectroscopic absorption properties led him to believe that the pigments were not identical.

Kühne in his celebrated work on "Optochemie" occupied himself somewhat again with the egg yolk pigment and called it Ontochrin or Lecithochrin. He did not try to isolate it free from nitrogen, but he did succeed in observing crystals. He again was careful to distinguish between the egg yolk pigment and the corpus luteum pigment. which he at this time considered as extraordinarily closely related to carotin.

The Lipochromes. Basing his work on the researches of Kühne. Krukenberg commenced a series of researches which extended from 1879 to 1886, the most important of which appeared in his "Vergleichende Physiologische Studien" [1] and especially in the paper, "Grundzuge einer vergleichenden Physiologie der Farbstoff und der Farben" which appeared in 1884. Krukenberg made an exhaustive study of what had been done on animal pigmentation and included under one head all those pigments which had previously been known as luteins. carotin, zoonerythrin (tetronerythrin) and Kühne's chromophanes, and called them lipochromes.

Krukenberg believed that carotin, the pigment of the carrot, was the best representative of the lipochrome coloring matters, and accepted Husemann's formula for carotin ($C_{18} H_{24} O$) as representing the chemical composition of the lipochromes.

In regard to the origin of lipochromes Krukenberg believed, "It is probable that in most cases they originate from fatty substances. for frequently, if not without exception, they occur in company with fat and allow themselves to easily go over into cholesterin-like bodies."

In 1885 Krukenberg [2] isolated a yellow lipochrome from the blood serum of the ox by extracting the serum with amyl alcohol. The solution showed two absorption bands, one enclosing the line F and the other lying between F and G. A year later Halliburton [3] reported that he extracted a yellow lipochrome from the blood serum of the pigeon, hen, dove and tortoise by means of alcohol. Halliburton reported an identical pigment in the body fat of these same animals.

MacMunn [4] was the next investigator of animal pigments, and like Krukenberg, he extended the classification lipochrome to include

1. Zoonerythrin (Tetronerythin):—Central, f. d. Medic. Wiss. 1879. Vergl. Physiol. Studien I Reihe, II Abth. s. 67-71; III Abth. s. 114-115; IV Abth. s. 30-35; V Abth. s. 87-94; II Reihe, I Abth. s. 165-167; III Abth. s. 135).
2. Sitz, ber. d. Jen. Gessel. f. Med. 1885.
3. Jour. Physiol. 7, p. 324 (1886).
4. Philos Trans. Roy. Soc. 177, p. 247 (1886).

the yellow constituent of chlorophyll or Hansen's "Chlorophyll Yellow." He believed that the lipochromes were chemically closely related to chlorophyll.[1]

MacMunn's greatest contribution to animal chromotology was in 1889.[2] The pigments of a great many marine animals, Crustacea, worms and sponges were examined and classified. Lipochromes were found abundantly, MacMunn drawing a distinction as to whether the lipochrome was a rhodophan or a chlorophan-like lipochrome.

In regard to some of the properties of the lipochromes MacMunn states, as did Krukenberg, that they are sensitive to light, both in the solid state and in solution, and yield in many cases cholesterin-like substances. He believed that many of the plant lipochromes were identical with the animal lipochromes.

It will be remembered that for a long time there were many followers of the view that a close relationship existed between carotin and cholesterol and that this view was only finally discredited by a study of the pure crystalline pigment.

Cotte[3] recently carried out an investigation in which he sought and claims to have shown that the lipochromes, both animal and vegetable are intimately associated with cholesterol. Cotte's results have been thoroughly disproved by Henze.[4]

Since the early work of Pouchet[5] and Maly[6] who distinguished between yellow and red crustacean lipochromes many investigators have classified the lipochromes according to their red or yellow color. Newbigin[7] in a recent investigation of the pigments of the skin, muscle and ovaries of the salmon, reports that he found two pigments present, a red and a yellow, which he claims he was able to separate from each other. Newbigin concluded from the color reactions of the pigments that the red pigment was a true lipochrome while the yellow pigment was not.

In regard to the yellow pigment, Newbigin says that, "It belongs to a group of pigments that are apparently exceedingly widely distributed in the animal kingdom, but which have been little investigated. They have been commonly confounded with the lipochrome pigments."

He extracted the pigment from the bright yellow body fat of a cow and found it to have properties identical with the yellow pigment

1. Jour. Physiol. 9, p. 1 (1888).
2. Quart. Jour. Micros. Sc. 30, p. 15 (1889).
3. Compt. Rend. Soc. Biol. 55, p. 812 (1903).
4. Zeit. Physiol. Chem. 41, p. 109 (1904).
5. Jour. d. l'Anat. de la Physiol. 1, 12, 10 (1876).
6. Sitz. d. k. Akad. d. Wiss. zu. Wein. 83 (1881).
7. D. Nöel Patton—Report of Inv. on Life Hist. of Salmon (1898), Article XV.

of the salmon with the exception that it was very little soluble in methyl alcohol, but dissolved readily in ether.

General Properties of the Lipochromes. It will not be out of place to give a brief summary here of the general characteristics and properties of the lipochrome pigments as found up to this time.

Lipochromes[7] may be classed as salve-like, yellow or red or orange colored residues, which have been obtained in needles or rhombic plates, where they have been crystallized. They are soluble in alcohol, ether, benzol, petroleum ether, amyl alcohol, chloroform, carbon bisulphide, ethereal oils and fats with a yellow or yellow-orange color. They are insoluble in cold and hot water and alkalies and dilute acids, but are soluble in alcoholic alkaline solutions and are unchanged when these solvents are heated. In alcohol or other solvents they are unstable, and readily bleach, as do the residues from these solutions. The bleach product is unknown, but it is certainly not identical with cholesterol. On addition of concentrated $H_2 SO_4$ or HNO_3, the lipochromes give a color change of blue-green-violet to brown. The color reactions are often interfered with by the presence of a small amount of foreign substance. The lipochromes generally give a blue-green coloration with a solution of iodine in potassium iodide. Spectroscopically the lipochrome solutions show two bands and sometimes three in the blue part of the spectrum, and again they sometimes show no bands at all.

The lipochromes may be extracted from the fresh or dried tissues in which they are found, by organic solvents, best by hot or cold alcohol, ether, petroleum ether, carbon bisulphide or chloroform, the choice of the solvent resting with whether some foreign pigment is present. When fat is present, the pigment may be heated with alcoholic alkali which will not saponify the lipochromes. The lipochromes can be extracted from the soap with ether, petroleum ether, or chloroform, either directly or after acidifying, or the lipochromes can be salted out of their alkaline soap solutions with sodium chloride, and the lipochromes obtained by extracting the precipitated soap with alcohol or ether.

The Lipochromes of Algae, Fungi, and Bacteria. While a wide distribution of the lipochromes has already been mentioned, a review of their literature would not be complete without mentioning their distribution in algae, fungi and bacteria.

1. Summarized from "Lipochromes" by Franz Samuely. Alderhalden's Biochemisches Handlexikon vol. 6, and Handbuch der Biochemischen Arbeitsmethoden, vol. 2.

Hansen[1] first showed the presence of lipochromes in algae and Tammes[2] has lately shown their presence in a large number of these plants. Zopf[3] has investigated the lipochromes of fungi and especially of bacteria, the first lipochrome-producing bacteria being pointed out by him.

The Lipochrome or Lutein of Egg Yolk. It will be readily agreed that while some order has been attained in classifying the widely distributed animal pigments, by means of the convenient and flexible classification "lipochromes," our knowledge of the animal pigments is far from being satisfactory when compared with the status of the orange and yellow plant pigments, the carotins and xanthophylls. The science of animal chromotology should accordingly be exceedingly grateful for the recent work of Willstätter[4] and Escher, on the lutein of egg yolk, the result of which has been to throw new light upon the constitution of the lipochromes of the higher animals and upon their relations to the carotins and the xanthophylls.

The main pigment of the yolk of hen's eggs was isolated in crystalline form by these investigators, and when in approximately pure condition showed sufficiently close agreement with the constitution of xanthophyll that the authors claim that the egg lutein on account of its melting point (195-196° C. corrected) is a true isomer of the crystalline xanthophyll of green plants. In all its other properties including its spectroscopic absorption bands, the egg lutein was identical with the crystalline plant xanthophyll.

It is worthy of note also that during the isolation of lutein a minor constituent was noticed which gave every indication of being closely related to carotin; but as it was present in very small amount compared with the xanthophyll it was disregarded.

In concluding the review of this investigation it will be important to mention that the authors state that one of them, i. e., Escher, is at present investigating the pigment of the corpus luteum which they state has been found to belong to the hydrocarbon or carotin group of pigments.[5]

1. Arbeit. Botan. Inst. Würzberg 3, 296 (1883).
2. Loc. cit.
3. Ber. Botan. Gessel. 9, 27 (1891).
4. Zeit. Physiol. Chem. 76, pp. 214-225 (1912).
5. Note—Since writing the above, Dr. Escher has published his investigations which show that the corpus luteum pigment is in every respect identical with the carotin of the carrot and of green plants. Zeit. f. Physiol. Chem. 83, p. 198 (1913).

The Physiological Relation Between Plant and Animal Lipochromes.

With the review of the chemical side of this problem complete, it yet remains to consider what has been shown in regard to relations other than chemical, between the animal and plant pigments whose properties are so nearly related and in many cases identical.

The literature has been found to be very brief on this point. Newbigin [1] gives a rather extensive consideration of this subject and attempts to explain the presence of the red and yellow pigments found by him in the salmon organism. While he considered the most obvious explanation to be that they were derived directly from the food, he found a number of difficulties in the way of the acceptance of such an explanation, the most important of which was that he was able to show the presence of but a trace of only the yellow pigment in the usual food of the salmon.

As to the possibility of transferrence of yellow pigments from one organism to another, Newbigin points out what he believes to be some evidence apart from the case of the salmon. He says, "Poulton [2] has shown by experiment that certain caterpillars derive their pigments from their food. Again it is not uncommon to find fat of sheep and cows dyed a deep yellow color. According to some authorities this occurs quite sporadically without known cause, while according to others, special foods, notably maize, are the important agents." Newbigin says in this connection, "I have examined the yellow pigment of maize, and compared it with the pigment from yellow fat. The maize pigment gives the lipochrome reaction faintly with H_2SO_4 distinctly with HNO_3 while the fat pigment gives no lipochrome reaction. In other respects, in tint, solubility, etc., the pigments closely resemble each other." Newbigin did not feel warranted to conclude from these experiments that all yellow pigments of animals are derived from their food, for with such a conclusion, he states, "It would be difficult to understand why such colored fat should not be universal in herbivorous animals, for all green parts of plants contain also a certain amount of yellow pigment."

It seemed to Newbigin, however, that a reasonable explanation for salmon, domesticated cattle and caterpillars would be to suppose that when they ingest a moderate amount of colored fat in their food, that they could utilize or eliminate the pigment, and so deposit colorless fat in the tissues; but when the ingestion of colored fat is in excess of the actual requirements as it so often is, especially with domes-

1. Loc. cit.
2. Proc. Roy. Soc. 54, p. 417; Nat. Sci. 8, p. 98.

ticated cattle, an elimination or utilization of the pigmented fat is impossible and fat colored with the pigment in a more or less modified condition is thus stored up.

There is abundant proof in this literature aside from the above speculations that animals are able to lay up fat soluble dyes in the organism and even eliminate them in the milk. Only recently Mendel and Daniels [1] have shown that Sudan III and other fat soluble dyes may be deposited in the organism in adipose tissue and bone marrow when introduced into the organism either dissolved in fat or when fed alone. When fed with fat or when fat was present in the alimentary tract the dyes entered the organism through the lymphatics in solution in fat, but when fat was absent, through the portal circulation dissolved in bile in which they are nearly all soluble. In the latter case the pigments did not pass beyond the liver unless fat was present to transport them, in which case only they were subsequently found in the blood. When fat stained food was fed to small animals (cats, rats, guinea pigs, etc.) in lactation, and in one case with a goat, the dye appeared in the milk shortly after the first feeding of the dye. The same authors feeding fifteen grams of Sudan III to a Holstein cow for three successive days were unable to detect the dye in the milk. The authors also made the interesting observation that stained fat does not traverse the placental barrier; the blood and foetus and fat of the young born of Sudan-stained female cats and rats were free from the dye.

Gogitidse [2] fed hog fat (100 grams per day) colored with Sudan III to a bitch and after two days found the dye in the milk. The body fat did not show this coloration so soon and then not so clearly, in fact only after long continued feeding of the stained fat.

Backhaus [3] studying the "Influence of Feed and Individuality on the Taste and Healthfulness of Milk," says that a number of plants influence the color of milk and butter. The same author conducted several pigment feeding experiments with cows. Negative results were obtained with respect to the milk when feeding Fuchsin, Bismark brown, and curcuma powder, although the feces showed the pigments abundantly. When feeding sodium fluorescin the urine was affected but not the milk. When feeding methyl violet, however, the author was able to show that this pigment was carried into the milk fat in a reduced condition so that on contact with the air and with the aid

1. Jour. Biol. Chem. 13, No. 1, p. 72 (1912).
2. Zeit. f. Biol. 45, 353 (1904).
3. Berichte, Landwirt. Inst. U. Königsberg 5 (1900).

of heat, the milk fat showed an intense blue coloration. The feces, however, showed the pigment in an unchanged condition.

Summary.

The foregoing review of the literature has shown that the great number of pigments that exist throughout the entire plant and animal kingdoms have long been of interest from a scientific standpoint. The pigments of botanical origin have been thoroughly and exhaustively investigated. This is especially true of the yellow pigments carotin and xanthophylls, and their chemical constitution and properties are now established.

The yellow and orange pigments of plants were at first classified in one group, and were called carotins, the name being derived from the pigment of the carrot, which was the first one investigated. A great many different names were given to this pigment as it was independently discovered in various plants but the identity of these pigments with the carrot pigment has now been established. It was eventually discovered that the carotins are always accompanied, especially in green plants, by a second great class of pigments which have been called xanthophylls, whose relation to carotin has but recently been established.

As the work on plant pigmentation developed, it was recognized that the general properties of a great many yellow pigments found in animals were similar to the so-called carotins. The first investigators classified these animal pigments under the name lutein, the name being derived from the pigment of the corpus luteum, which was the first one investigated. The name lutein was extended by the animal chromotologists to include the carotins of plants and its related pigments. Later, when the animal luteins had become generally recognized by their association with fat, the name lutein was changed to lipochrome and this designation was also extended to include all similar pigments of both plants and animals.

The classification of the plant and animal pigments which is at present generally accepted is to restrict the names carotin and xanthophylls to the two great classes of yellow plant pigments, and to include under the name lutein or lipochrome only those yellow pigments which are considered to be of animal origin.

The most recent work in the field of animal chromotology has shown that the luteins can also be subdivided into carotin and xanthophyll groups depending on their chemical relation to the carotin or xanthophylls of plant origin. Accordingly Schunck [1] has shown the

1. Loc. cit.

spectroscopic identity of the egg yolk pigment with a xanthophyll which he isolated from the yellow daffodil, the nasturtium, and green leaves. Willstätter and Escher [1] have confirmed this with a chemical analysis of the egg yolk pigment, showing it to be a true isomer of the crystalline xanthophyll of green plants; they have called it xanthophyll B. Escher [2] has recently published his investigation showing that the pigment of the corpus luteum is identical in chemical composition and properties with the carotin of green plants.

These recent discoveries have opened the way for an extension of such investigations to other yellow animal pigments whose isolation is rendered much more difficult by their association with very large quantities of fat and other substances. These discoveries have also raised the question whether any relation other than chemical exists between the yellow animal and plant pigments. This question has never been investigated. The investigations which will be reported in the succeeding papers are the first to show that there is a definite relation other than chemical between the yellow plant and animal pigments.

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2. Loc. cit.

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61. Willstätter and Escher: Zeit. f. Physiol. Chem. 64, p. 74 (1910); 76, pp. 214-225 (1912).
62. Willstätter and Meig: Ann. d. Chemie 355, p. 1 (1907).
63. Wittich: Arch. .. Path. Anst. 27, p. 573 (1863).
64. Wirth: Dissert. Erlangen (1891).
65. Zeise: Lieb. Ann. 62, p. 380 (1847); Ann. Chem. Phys. (3) 20, p. 125 (1847).
66. Zopf: Ber. der d. Botan. Gessel. 9, p. 27 (1891).

CAROTIN—THE PRINCIPAL NATURAL YELLOW PIGMENT OF MILK FAT—PART II.*

Chemical and Physiological Relations of Pigments of Milk Fat to the Carotin and Xanthophylls of Green Plants.

LEROY S. PALMER AND C. H. ECKLES

For a number of years the great variety of yellow animal pigments have been classified under the general name lipochrome. Recent investigations by Willstätter and Escher,[1] and by Escher [2] have shown, however, that some of these pigments are in reality very closely related or identical with the carotin or xanthophyll pigments of plants. Willstätter and Escher have analyzed the pure lutein of egg yolk and found it to be isomeric with the crystalline xanthophyll of green plants; and Escher has identified in the same manner the lipochrome of the corpus luteum as a true carotin. The isolation of both pigments was attended with great difficulty. In the case of the egg yolk pigment the yolk of 6,000 hen eggs yielded only four grams of crude crystalline pigment, while in the case of the corpus luteum pigment less than 0.5 gram of crystals were obtained from 10,000 cows' ovaries.

The natural yellow pigment of butter is the most commonly observed of all animal lipochromes. It is also more important from a commercial standpoint than any other animal pigment; the public judges the richness of dairy products by their yellow color, and demands that butter especially shall have a standard shade of yellow. The pigment of butter fat, however, has been the least investigated of all animal lipochromes. Thudichum's [3] classical investigation included the pigment of butter fat under the general classification lutein, which he proposed. No other study of the butter fat pigment has

1. Zeit. f. Physiol. Chem. 76, pp. 214-225 (1912).
2. Zeit. f. Physiol. Chem. 83, p. 198 (1913).
3. Proc. Roy. Soc. 17, p, 253 (1869).

* See Res. Bul. No. 9, p. 312, for statement of co-operation with U. S. Dept. of Agriculture.

been reported. It is usually classified in the current text books,[1] however, according to Krukenberg's classification of lipochromes.

In view of the commercial importance attached to the butter fat pigment and especially in view of the results of the most recent investigations in this field, it was recognized that a thorough investigation of this pigment would be of great value both from a scientific as well as a practical standpoint.

The present investigation was therefore undertaken for the purpose of classifying the butter fat pigment as a true lipochrome and also with respect to its relation to the carotin and xanthophylls of green plants. It was also the purpose of the investigation here recorded, to gather as much information as possible relative to the influence of certain factors upon the color of butter, among which may be mentioned the character of the ration and the breed of the cow.

METHODS OF ISOLATION.

The statement is frequently met in the literature[2] and in the text books and works[3] on oils and fats, that the pigment of butter or butter fat appears in the unsaponifiable extracts along with cholesterol and other substances.

A number of methods for obtaining the unsaponifiable matter of butter fat are available and several were tried. The method finally adopted for the isolation of the crude pigment was to saponify the butter fat with a twenty per cent solution of alcoholic potash, using 2 c. c. for each gram of fat. Saponification was allowed to continue for one half to one hour at the temperature of the boiling solution. The soap was dissolved in three volumes of distilled water. After cooling, the solution was shaken with an equal volume of pure ether in a separatory funnel. The extraction was repeated with a fresh volume of ether equal to one-half the volume of the soap solution. It was found that this procedure would leave the soap colorless, if no aldehyde resins had formed during saponification or none of these colored bodies had been present in the alcoholic potash previous to its addition to the fat. The ether extract containing the pigment and other unsaponifiable matter was now freed from alkaline soap, by shaking many times with excess water, carefully at first to avoid

1. Such as Hammarsten, "Text Book of Physiological Chemistry": and Schaefer, "Text Book of Physiological Chemistry", etc.
2. Kirsten: Zeit. Nahr. Genuss. 5, p. 833 (1903).
3. Lewkowitsch: "Oils, Fats and Waxes." Vol. I, p. 371, (1909 Edition).

emulsions, and more vigorously with subsequent washings. When the wash water no longer reacted alkaline toward phenolphthalein, the ether was either dried over fused $CaCl_2$ or, after standing several hours, decanted from the precipitated moisture. The ether was then evaporated, leaving a salve-like residue of various tints of yellow to orange to red, depending upon the amount of fat used and the depth of its original color.

Although the methods of study eventually adopted did not make the procedure necessary, it was found possible to completely free the pigment from its chief impurity, i. e., cholesterol, by means of the digitonin method of Windaus [1] for the quantitative estimation of cholesterol. A hot one per cent solution of digitonin in ninety per cent alcohol, when added to an alcoholic solution of the unsaponifiable residues from butter fat, completely precipitated the cholesterol as a colorless compound, leaving the pigment in solution. This solution was still contaminated, however, with traces of fat and lecithin decomposition products.

GENERAL PROPERTIES OF THE BUTTER FAT PIGMENT.

The unsaponifiable residues from butter fat, either crude or freed from cholesterol by digitonin, were readily soluble in hot alcohol and in ether, chloroform, petroleum ether, etc. with a golden yellow color; and in carbon bisulphide with a color which varied, according to the concentration, from a red orange to a blood red color. The freshly prepared crude, and also, the cholesterol-free residues usually gave an instantaneous but transient purple color on adding a drop of concentrated sulphuric acid, a light green color quickly changing to a greenish blue color on adding a drop of concentrated nitric acid, and a dark blue color with the combined acids. These color reactions were usually shown a little clearer by the cholesterol free residues. Small amounts of impurities often interfered with the color reactions; in fact they were often rendered negative by some slight decomposition of the pigment which was not apparent in the intensity of the color. This was due to the fact that the crude samples of butter fat pigment were very unstable, quickly bleaching in the air, especially with the aid of heat in the presence of a little water. It was necessary therefore to take great care to have the ether solutions of the pigment as free as possible from water before evaporation, or to transfer the pigment to some solvent such as petroleum ether, which does not absorb water so readily.

1. Zeit. f. Physiol. Chem. 65, p. 110 (1909).

In addition to the above properties it was found that all fat-free, but not necessarily cholesterol-free solutions of the pigment showed spectroscopic absorption bands. In alcohol two sharp bands were exhibited in the blue part of the spectrum; and in carbon bisulphide these bands were nearly always accompanied by a third faint band in the violet, which was now visible on account of the general shifting of the bands toward the red end of the spectrum. The unstable character of the butterfat pigment required that the isolation be carried out as rapidly as possible in order to preserve all the characteristic properties of the pigment. This was especially true for the study of the absorption spectra. Fifteen to thirty grams of fat were found to yield sufficient pigment for a spectroscopic study. The use of large quantities of fat (300 to 1,000 grams) always led to unsatisfactory results.

The general properties of the butter fat pigment show that it is to be classed as a true lipochrome. The chemical relation of the pigment to the carotin and xanthophylls of green plants remains to be shown.

METHODS OF IDENTIFICATION.

The nature of the substances with which the butter fat pigment is associated at once precluded its isolation in sufficient quantity to establish its chemical composition and molecular weight. It was therefore necessary to adopt other methods of identification which would be sufficiently accurate and characteristic that the final results could not be mistaken.

The methods that were adopted were, (1) a study of the spectroscopic absorption properties, (2) a study of the relative solubility properties, (3) a study of the adsorption properties with respect to calcium carbonate; (4) an attempt was also made to study the crystalline form.

Before giving the results of our studies, some discussion will be given of the relative solubility and adsorption properties of carotin and the xanthophylls.

RELATIVE SOLUBILITY OF CAROTIN AND XANTHOPHYLLS.

M. Tswett[1] was the first one to publish a comprehensive statement in regard to the relative solubility of the plant pigments in

1. Ber. der. Deut. Botan. Gessel. 24, pp. 316, 384 (1906).

different solvents. He classified the solvents into three groups as follows according to their relation to the leaf pigments.

"1. Alcohols (methyl, ethyl, amyl), acetone, acetaldehyde, ether, chloroform: These solvents, acting on freshly cut up or dried leaves dissolve out all pigments equally and completely."

"2. Petroleum ether or petroleum benzin: This solvent, acting on fresh leaves finely ground with sand or emery, takes on a more less yellow appearance, which is especially due to carotin, but contains also other pigments. Dried leaves (at a low temperature) likewise give up their carotin to this solvent, and in somewhat purer condition."

"3. Benzol, xylol, toluol, and carbon bisulphide: These solvents set intermediately between the first two groups."

Willstätter and Mieg [1] a little later approached the same problem from another standpoint and showed that the methods used by Kraus [2] and Sorby [3] for demonstrating the presence of more than one pigment in green plants, when properly applied could be made characteristic properties of carotin and xanthophylls. Kraus shook his alcoholic extracts of green leaves with petroleum ether and found that the green pigment went into the petroleum ether leaving the alcoholic solution yellow. Sorby shook his alcoholic extracts with carbon bisulphide and found that the latter solvent contained the green pigment while the alcohol was left yellow. Wilstätter and Mieg, applying these tests to the isolated carotin and xanthophyll pigments obtained the following results:

"1. If methyl alcohol is added to a petroleum ether solution of carotin so that the liquids do not mix, the carotin will remain for the greatest part in the petroleum ether layer, the alcohol layer being only slightly colored. If a trace of water is added, the methyl alcohol layer will become colorless. The same phenomenon occurs with ethyl alcohol, and one can start with an alcohol or benzol solution and show the same thing."

"2. If carbon bisulphide is added to an alcoholic carotin solution and a little water added, the carbon bisulphide will separate and will quantitatively contain the carotin."

"3. If an alcoholic solution of the xanthophylls is mixed with petroleum ether and the liquids separated with a little water, by far the greatest portion of the xanthophylls will be found in the alcohol layer."

1. Ann. der. Chemie 355 p. 8 (1907).
2. Flora, p. 155 (1875).
3. Proc. Roy. Soc. 21, p. 456 (1875).

"4. If an alcoholic solution of xanthophylls is mixed with carbon bisulphide and the solvents separated with water, the xanthophylls will be distributed between both layers."

Schunck,[1] using Sorby's method, showed that if alcoholic solutions of xanthophylls are repeatedly shaken with carbon bisulphide all the xanthophylls with spectroscopic absorption properties can be extracted.

Tswett [2] studying this question again in 1911 states that, "According to a well known rule, organic compounds are best soluble in solvents of similar composition," and concludes that carotin, a hydrocarbon, is therefore much more readily soluble in the hydrocarbons of the aliphatic and cyclic group than in alcohols, a point well illustrated by the above experiments of Willstätter and Mieg. Continuing, Tswett states, "If one therefore shakes an eighty to ninety per cent alcoholic solution of carotin with petroleum ether, the pigment goes almost completely into the petroleum ether layer." "A pigment which in the above mentioned two phase system occupies the lower alcoholic layer, is therefore not a carotin." It may be added in the light of Willstätter and Mieg's investigation that if the original solution before differentiation was a mixture of carotin and xanthophylls, the lower alcoholic layer will contain the xanthophylls.

ADSORPTION PROPERTIES OF CAROTIN AND XANTHOPHYLLS.

Considering now the so-called adsorption properties of the pigments, we find that this striking characteristic was discovered and elaborated by Tswett.[3] This investigator found that by shaking a perfectly anhydrous petroleum ether or carbon bisulphide solution of the mixed pigments of green plants with an excess of dry calcium carbonate, Inulin or Saccharose, all the pigments will be completely adsorbed by the material with the exception of the carotin, which can be readily washed out of the material with the free solvent. In the case of petroleum ether solutions the green colored mass can now be completely freed from all its pigments by means of petroleum ether containing ten per cent absolute alcohol. If the resulting solution is now shaken with eighty per cent alcohol the petroleum ether layer will contain the chlorophyll pigments and the alcohol layer the xanthophylls. "If to the petroleum ether solution of the mixed pigments

1. Proc. Roy. 72 (1903).
2. Ber. der. Deut. Botan. Gessel. 29, p. 630 (1911).
3. Ber. der. Deut. Botan. Gessel. 24, p. 316 and 384 (1906).

is added adsorption material only sufficient to destroy the fluorescence, both the carotin and xanthophylls remain in solution," and can be separated by means of a differentiation between the petroleum ether and eighty per cent alcohol, or, "By treating the solution with more adsorption material, after pouring it away from the first, and the xanthophylls then freed from combination with the adsorption material by means of alcoholic petroleum ether." In addition to the above, Tswett made the interesting discovery that the pigments which are adsorbed by the various materials suggested, can to a certain extent displace one another in the adsorbing material. As an example one finds that, "If a petroleum ether solution of the mixed pigments is filtered through a column of adsorption material (such as $CaCO_3$ packed tight in a glass tube) the pigments will be separated from one another from top to bottom in differently colored zones, proportionately to their degree of adsorption." This separation will be complete if a stream of pure solvent is put through the column after the pigment has been adsorbed in the upper part of the column. As stated by Tswett, "Like the rays of light in the spectrum, so the different components of a pigment mixture are actually separated in the $CaCO_3$ column, and may thus be qualitatively estimated." Tswett calls such an experiment a "chromotogramm." He found carbon bisulphide to be one of the most useful solvents for a chromotographic analysis, on account of the brilliant color which all pigments show in this solvent.

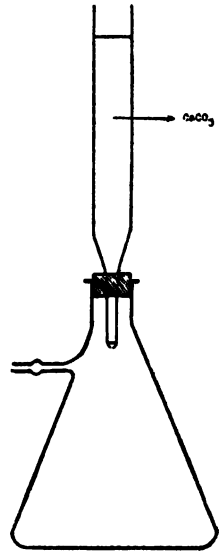

FIGURE I.

In describing the technique for the chromotographic analysis, the author mentions the following essential points. A very finely divided material with not too strong adsorption properties should be used for the adsorbator. ($CaCO_3$ was found to answer these qualifications best.) A glass tube is now prepared 10 to 20 m. m. in diameter and 15 to 20 c. m. long, one end of which is drawn out to a narrow diameter, at which end the opening is fused in a little to form a base for the deposition of the adsorbing material. A small

piece of cotton is placed in the small end of the tube and the perfectly dry $CaCO_3$ poured in and firmly tamped down to a homogenous texture. The chromotographic apparatus may now be arranged according to Figure I, and the filter flask attached to the suction pump. The $CaCO_3$ is now moistened with a little of the solvent to be used, (this is very necessary) and a certain amount of the liquid which is to be studied poured on the $CaCO_3$. A stream of pure solvent is subsequently established and the different adsorption zones will then spread out and reach their definite maximum differentiation. All unadsorbed substances will be completely washed away and, "Substances which form truly dissociable adsorption compounds with the $CaCO_3$ pass slowly, 'ringwise' through and can be taken up each by itself at the mouth of the tube."

Carotin and Xanthophyll of Green Plants.

After selecting the methods of differentiation and characterization to be applied to the milk fat pigment, it was considered desirable to ascertain whether they were sufficiently characteristic for a complete identification, should the milk fat pigment be found to be either a carotin or a xanthophyll. The following experiment was accordingly carried out.

About fifteen grams of air dry and finely divided alfalfa hay which had a deep green color, was let stand for several days, with shaking, under pure carbon bisulphide. The resulting deep olive brown fluid was concentrated to about 25 c. c. at a low temperature. A glass tube about eight inches long and one-half inch in diameter, the last two inches of which were drawn out to a small opening, was now filled and packed with pure $CaCO_3$, which had been previously dried for two hours at 150° C. The $CaCO_3$ was tamped in a small portion at a time by means of a small cotton wad and a heavy glass rod. The small end of the tube was now inserted through a one hole rubber stopper and fitted tightly into a side neck test tube. The apparatus was then attached to a suction pump. A stream of pure carbon bisulphide was passed through the column. When the $CaCO_3$ had become thoroughly moistened, 2 to 3 c. c. of the alfalfa extract was poured into the top of the column, vigorous suction being maintained all the time. When the extract had passed entirely into the $CaCO_3$ and occupied about one inch of the column, a stream of pure carbon bisulphide was run through. As the pigment passed through it differentiated itself into a number of green and yellow zones, the least

adsorbed pigment at the bottom of the differentiation having a rose color. The stream of carbon bisulphide was continued until the rose colored solution began to drop from the lower end of the tube. The appearance of the column at this time is shown in Figure II. The stream of carbon bisulphide was now continued until the rose colored zone had entirely passed through.

According to Tswett this zone contained only carotin. The beautiful rose or red orange solution that was obtained was studied as follows: It was first examined in the spectroscope where it showed two distinct bands, and a faint third one. (See Table I.) The carbon bisulphide solution was then evaporated. The residue was on orange yellow solid. A portion of it gave a beautiful blue coloration with concentrated H_2SO_4.

The remainder of the residue was dissolved in hot ninety-five per cent alcohol, and the phystosterol which precipitated out on cooling filtered off. The filtrate was divided into two portions, petroleum ether (b. p. 30-50° C) being added to one and pure carbon bisulphide to the other. On separation of the respective solvents with a little water, the pigment was found quantitatively in the petroleum ether and carbon bisulphide layers respectively. The pigment was again put into alcohol, and in this solvent showed two strong absorption bands and end absorption. (See Table I.)

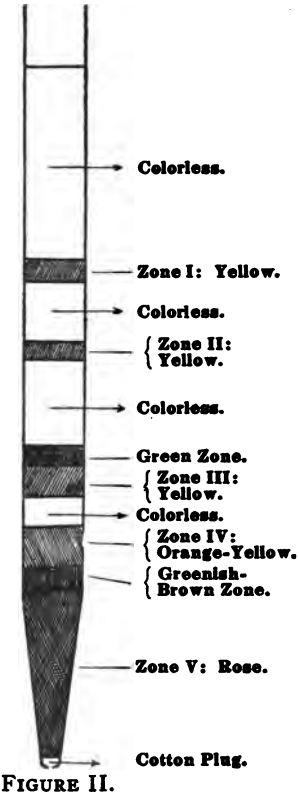

FIGURE II.

Potassium hydroxide was now added to the alcoholic solution and the solution boiled for several hours. The alkaline solution was diluted with three volumes of distilled water and extracted with an equal volume of ether. The ether completely and readily extracted the pigment, showing it to be unsaponifiable. The golden yellow ether solution was washed free from alkali with water and evaporated to dryness. The residue was taken up with alcohol. This solution showed only two bands. (See Table I.)

The alcoholic solution was now tested again for its relative solubility toward petroleum ether (b. p. 30-50° C) and carbon bisul-

phide. The pigment was again found quantitatively in the petroleum ether and carbon bisulphide. A portion of pigment was again put into alcohol and the solution made strongly alkaline with solid sodium hydroxide. On addition of much sodium chloride to this solution the pigment was not precipitated. The remainder of the pigment was now dissolved in carbon bisulphide, after evaporation of the solution (petroleum ether), and the carbon bisulphide solution filtered through the $CaCO_3$ column again. It passed through unadsorbed as a rose colored zone.

TABLE 1. ABSORPTION (a) BANDS OF ALFALFA CAROTIN

	In CS_2	In C_2H_5OH	In C_2H_5OH (after saponification)
Band I	225-245	256-275	256-277
Band II	259-279	300-320	303-320
Band III	300-320	345-...	.....

(a) Note: It should be noted that all spectroscopic measurements, both this and subsequent ones, were made according to an arbitrary scale which was attached to the spectrometer. This scale was always set to a fixed standard before studying each pigment, the standard being produced by a sodium flame with the spectrometer slit closed to furnish the narrowest possible line. The spectrometer was equipped with a crown glass prism and lenses and had a narrow dispersion.

The study of the alfalfa carotin showed conclusively that its adsorption, spectroscopic and solubility properties were clear and characteristic and were unchanged by boiling in alcoholic potash. It was also shown that the pigment could not be salted out of its sodium alcoholate solution with common salt when the pigment was free from fat. If the solution had contained much soap the pigment would in all probability have been precipitated with the soap in the salting out process. The object of the test was to see whether this was or was not a characteristic test for a comparatively pure carotin. Newbigin[1] claimed to have found a true lipochrome which could be salted out of its alkaline solution.

1. D. Nöel Paton. "Investigations on Life History of the Salmon." 1898, Art. XV. p. 159.

After studying the carotin pigment, more carbon bisulphide was run through the column shown in Figure II. until the lower green zone was washed out. This was removed from the test tube and the stream of carbon bisulphate continued until the two least adsorbed orange-yellow zones had been washed out. The carbon bisulphide solution of these pigments had a golden yellow color with a faint green tinge. Before the spectroscope the solution showed two chlorophyll bands in the red, and three very clear bands in the blue. (See Table 2.)

The carbon bisulphide was evaporated off and the residue dissolved in hot eighty per cent alcohol, the phytosterol which precipitated on cooling being filtered off. The alcoholic filtrate was now extracted with petroleum ether (b. p. 30-50° C), which took up some green color. This was continued until no more green was extracted. The golden yellow alcoholic xanthophyll solution was examined in the spectroscope. No chlorophyll bands were visible but only two strong bands in the blue and sharp end adsorption.

The alcoholic solution was now saponified with potassium hydroxide and the diluted soap extracted with ether in the usual way. The color immediately went into the ether, the separation being complete with one extraction. After washing free from alkali the ether was evaporated. A portion of this residue as well as a portion of the residue of the solution before saponification, showed a beautiful greenish blue coloration with concentrated H_2SO_4. The remainder when dissolved in alcohol, gave a golden yellow solution showing two bands and end absorption. (See Table 2.)

TABLE 2. ABSORPTION BANDS OF ALFALFA XANTHOPHYLLS

	In CS_2	In C_2H_5OH	In C_2H_5OH (after saponification)
Band I	232-250	265-282	265-286
Band II	273-293	306-326	306-326
Band III	312-330	357-. . .	355-. . .

A little HCl added to a portion of the alcoholic solution gave no blue coloration. Solubility tests on the saponified pigment showed that petroleum ether extracted no color from an alcoholic solution on dilution with a little water, while carbon bisulphide extracted about an equal portion.

These tests showed conclusively that this pigment belongs to the α group of xanthophylls; and that its adsorption, solubility and spectroscopic properties which are characteristically different from those of carotin, are unaltered by treatment with alcoholic potash.

The remaining xanthophyll pigment was so firmly held in combination with the $CaCO_3$ that carbon bisulphide would not wash it out. A stream of ten per cent alcoholic petroleum ether was therefore run through the column, washing out all the remaining pigments. The resulting solution showed only faint absorption bands, indicating that the pigments noted above, i. e. carotin and xanthophyll α, are the principal yellow pigments of the alfalfa hay.

IDENTIFICATION OF THE PIGMENT OF BUTTER FAT.

With the properties of carotin and xanthophylls well established, attention was next directed to the butter fat pigment. The following experiments were carried out, the results of which are very striking.

Experiment 1.

Fifteen grams of very yellow butter fat from a Jersey cow who was on fresh, green, fall grass was saponified in the usual way with alcoholic potash, taking great care to avoid the presence or formation of the colored alcohol decomposition products, the aldehyde resins. After dilution the soap was extracted with ether. The ethereal extract was washed free from alkali with distilled water and evaporated into ninety-five per cent alcohol. An equal volume (100 c. c.) of petroleum ether (b. p. 30-50° C.) was now added to the alcoholic solution and just enough water to cause a separation of the alcohol and petroleum ether. The golden yellow petroleum ether layer which resulted, contained practically all the color. The alcohol layer was drawn off and extracted with fresh volumes of petroleum ether until only a trace of color went into the petroleum ether layer. All the petroleum ether extracts were then combined and extracted with eighty per cent alcohol. A mere trace of color went into the alcohol. The alcohol solutions were combined.

The Petroleum ether solution.—This contained by far the greatest part of the total pigment. The solution was evaporated quickly at a temperature below 50° C., leaving a red oily residue which instantly dissolved in carbon bisulphide with a deep red orange color. After adjusting the concentration for the 10 m. m. cell, so that the bands

were all plainly visible, this solution showed three distinct absorption bands. (See Table 3.)

The carbon bisulphide solution was evaporated to dryness at the lowest possible temperature, the residue taken up in 50 c. c. of hot ninety-five per cent alcohol and hot one per cent digitonin in ninety per cent alcohol added until no more precipitate came down. The digitonin-cholesteride was filtered off, the filtrate evaporated to dryness, and the residue dissolved once more in carbon bisulphide. The solution still showed the three bands. On evaporation it left a golden yellow oil which solidified on cooling to a reddish yellow salve. Concentrated H_2SO_4 added to the residue gave a blue green color which slowly changed to a purple color.

The alcoholic solution.—This was evaporated to dryness. When very concentrated it showed a little color, and the carbon bisulphide solution of the residue had a light orange color when it had a volume of 1½ c. c. When viewed in a 25 m. m. cell this solution showed three absorption bands. (See Table 3.)

TABLE 3. ABSORPTION BANDS OF BUTTERFAT PIGMENTS.

	Petroleum ether soluble pigment In CS_2 solution		Alcohol soluble pigment in CS_2 solution
	Crude	Free from Cholesterol	
Band I	222-240	224-242	229-247
Band II	257-276	260-277	269-288
Band III	299-319	301-316	309-328

The striking results of this experiment were the remarkable similarity of the solubility and spectroscopic properties of the main butter fat pigment to carotin, and the indications of a secondary minor constituent of the butter fat pigment, whose solubility and spectroscopic properties were strikingly similar to xanthophyll.

It at once became evident that should these observations be confirmed, it would be not only profitable, but essential to ascertain whether the presence of secondary xanthophyll-like pigments is normal to butter fat under other conditions of coloration, such as in light colored butter fat, colostrum butter fat and other conditions.

That the presence of a secondary pigment in the fat under investigation was confirmed and its character more clearly identified is shown by the following experiment.

Experiment 2.

Fifteen grams of the fat was treated as in Experiment 1. In addition the carbon bisulphide solution of the unsaponifiable ether extract was filtered through a column of $CaCO_3$ in a manner identical with the chromotographic experiment with alfalfa hay. As far as could be detected with the eye all the pigment passed quite rapidly through as an unadsorbed rose colored zone, which spread out considerably in its passage through the column but showed no differentiation into zones. The absorption bands of the filtered pigment were identical with the bands of carotin.

After the carbon bisulphide had washed out all the pigment and was passing through colorless, a stream of petroleum ether containing ten per cent alcohol was run through the column. As it passed through it gathered a zone of yellow color, leaving the column pure white. This pigment was collected at the mouth of the tube, its solution evaporated, and the residue dissolved in carbon bisulphide. The light-orange colored solution showed two strong absorption bands and a third fainter one.

The carbon bisulphide solution of the main pigment was now evaporated to dryness and the residue dissolved in ninety-five per cent alcohol. The alcohol was diluted with water to an eighty to ninety per cent solution and extracted with petroleum ether (b. p. 30-50° C). The bulk of the pigment went into the petroleum ether and a second extraction with fresh petroleum ether took out still more pigment. A third extraction with fresh petroleum ether, however, left the alcohol layer considerably more colored than the petroleum ether layer. The alcohol layer was now evaporated and the residue dissolved in carbon bisulphide, giving an orange yellow solution which showed three strong absorption bands. There seemed to be three or four times as much of this pigment as of the xanthophyll which had been adsorbed by the $CaCO_3$ in the chromotogramm, and together they probably amounted to ten per cent of the total pigment.

All the xanthophyll pigments were now combined (they were all in carbon bisulphide solution) and the resulting solution analyzed by means of a chromotogramm. As the orange-yellow solution was washed through the column by a stream of carbon bisulphide it took on the appearance as shown in Figure III. Zones two and three were collected together, and showed three absorption bands. (See Table 4.) Their solution was evaporated and the residue dissolved in petroleum

ether. Only a part of the residue would dissolve readily, and the remainder only on addition of a little absolute alcohol. Eighty per cent alcohol extracted a large part of the color from the alcoholic petroleum ether solution. The portion which remained in the petroleum ether was transferred to carbon bisulphide in which it showed three absorption bands. (See Table 4.) The portion extracted by the eighty per cent alcohol was also transferred to carbon bisulphide. The latter solution showed three absorption bands but the third was faint and was not measured. (See Table 4.)

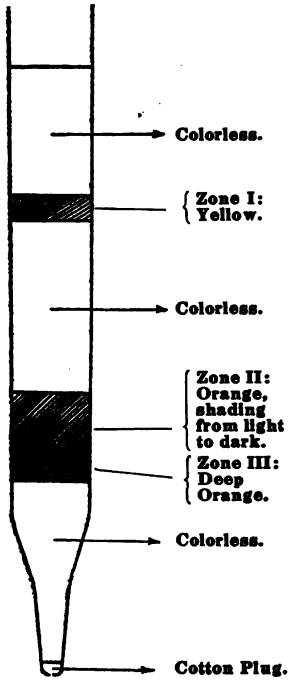

FIGURE III.

Alcoholic solutions of both portions of pigment showed no color change on the addition of a little concentrated HCl. There was also no effect on the absorption bands.

Zone I of the chromotogramm was of a pure yellow color. It was completely adsorbed by the $CaCO_3$ with respect to CS_2 and was evidently the same pigment which had been adsorbed in the first chromotogramm of the combined carotin and xanthophyll-like pigments. A stream of alcoholic petroleum ether readily washed it out of the column as it did in the first chromotogramm. In carbon bisulphide solution the pigment had a light orange color, and showed two brilliant absorption bands and a third fainter one. (See Table 4.)

The pigment showed the three bands in alcoholic solution as well as in carbon bisulphide. When in alcohol, it gave no color reaction with a little concentrated HCl, and there was also no immediate effect upon either the intensity or position of the absorption bands. In the solid state this pigment gave a transient greenish-blue color with concentrated H_2SO_4.

For the sake of comparison with the carotin bands of the alfalfa hay, the alcoholic solution of the main butter fat pigment was examined in the spectroscope. The results are given in Table 5.

TABLE 4. ABSORPTION BANDS OF BUTTER FAT XANTHOPHYLLS SHOWN IN FIGURE III.

	Zone I. CS_2 solution	Zones II & III C_2H_5OH solution	Zones II & III	
			(P. ether sol. Part) In CS_2	(Alcohol sol. Part) In CS_2
Band I	232-249	263-280	230-249	231-250
Band II	271-288	306-325	268-289	272-240
Band III	313-330	355-. . .	312-330	Not measured

TABLE 5. COMPARISON OF BANDS OF CAROTIN OF ALFALFA AND BUTTERFAT. (C_2H_5OH SOLUTION).

	Alfalfa Carotin	Butterfat Carotin
Band I	256-275	256-274
Band II	300-320	298-312
Band III	345-. . .	345-. . .

The result of this experiment was not only to confirm the remarkable similarity of the main butter fat pigment of this particular fat to the carotin of alfalfa hay, but also to confirm the presence of a secondary constituent practically identical with the xanthophylls. In addition it was found that these xanthophylls, like the xanthophylls of alfalfa hay, could be chromotographically separated into three constituents. The two main constituents seemed to be closely related to carotin in adsorption properties so that their presence could not be detected in the presence of a large amount of carotin until first separated from the main pigment with the aid of their relatively greater solubility in alcohol with respect to petroleum ether. The third constituent of the secondary group was more nearly related to a true xanthophyll in all its properties, including its adsorption by $CaCO_3$. When classified with respect to the action of their alcoholic solutions toward a little concentrated HCl all the xanthophyll pigments apparently belong to the group which Tswett calls α xanthophylls.

STANDARDIZATION OF ABSORPTION BANDS OF CAROTIN AND XANTHOPHYLLS.

Before proceeding further it may be well to make a few statements in regard to the spectroscopic properties of the various pigments which have been under consideration and which are still to be considered. It will be obvious that measurements of absorption bands could not be made in this work with the accuracy attained by Willstätter and his collaborators who used solutions of standard strength and a spectroscope of great exactness. Small differences in measurement among xanthophylls or between carotins should accordingly be disregarded. It was merely attempted in every case to secure solutions of such concentration that the bands were of as nearly the same intensity as could be detected with the eye before making measurements. It was not always possible to use the same thickness of cell to secure the required intensity of the bands, small amounts of pigment naturally requiring a greater depth of solution than large amounts.

In order to have standard spectroscopic properties for future comparison, carotin and xanthophylls were extracted from the carrot, and a careful study made of the spectroscopic properties of each pigment.

A grated carrot was boiled in water for about two hours, the water squeezed out of the pulp and the pulp dried on the steam bath. It was pulverized and extracted with ether in a Soxhlet extractor until colorless. The ether was evaporated into ninety-five per cent alcohol at a low temperature. The resulting solution was diluted with a little water to eighty per cent and the pigment carefully separated between petroleum ether (b. p. 30-50° C) and the eighty per cent alcohol. Both portions of the pigment were carefully transferred to pure carbon bisulphide and the solutions adjusted for the 10 m. m. cell until all bands were distinct and as nearly as possible of equal intensity. With the spectrometer set at the sodium line standard the positions of the absorption bands were standardized. The color of the solutions was measured also, by means of the Lovibond tintometer. (See Figure V.)

This data is given in Table 6.

TABLE 6. SPECTROSCOPIC STANDARD OF CAROTIN AND XANTHOPHYLLIS. (FROM THE CARROT.)

Pigment	Layer	Color			Absorption Bands		
		Yellow	Red	Light	Band I	Band II	Band III
In CS_2							
Carotin	10 m.m	26.0	5.5	1.0	225-242	261-278	301-319
Xanthophyll	10 m.m	17.0	4.4	0.5	233-253	272-291	312-330
In C_2H_5OH							
Carotin	10 m.m	....	...	...	257-275	303-318	345-364
Xanthophyll	10 m.m	....	...	...	263-280	305-325	355-. . .

It will be noticed that the relative position of the bands of carotin and xanthophylls is more characteristic in carbon bisulphide than in alcohol. On this account, nearly all subsequent spectroscopic studies were made in carbon bisulphide solution.

It will be noticed that both pigments showed three bands. The difference in the color of the carbon bisulphide solutions of the carotin and xanthophyll pigments when showing bands of equal intensity is also especially noteworthy. To the eye, the carotin solutions are a deep red orange while the xanthophyll solutions are a much purer orange color. It is worthy of mention that this characteristic difference was so noticeable throughout all this study, that in all cases the color of the carbon bisulphide solution of unknown pigments was recorded in connection with the measuring of the absorption bands.

CHARACTER OF THE PIGMENTS IN DIFFERENT BUTTER FATS.

It was stated previous to the confirmation of the presence of secondary xanthophyll-like pigments in the particular butter fat studied, that if the presence of these pigments could be confirmed it would necessary to establish such a fact as either characteristic of all butter fat or as incidental only to the particular fat studied.

A wide variety of fats of different color, from different breeds of cows and produced under widely different conditions of feeding, etc., were available for this study. The technique of these experiments was identical with that used for the high colored fat from the Jersey cow on grass recorded in Experiments 1 and 2 above.

The Pigments of Light Colored Fats.—The character of the pigment in three light colored fats was tested. The fats represent three subsequent periods in a feeding experiment of a pure bred Ayrshire cow in which the color was practically eliminated from the butter fat. (See Table 13 of feeding experiments for record of this experiment.) The color of the fat and the character of the pigments are shown in the following Table 7.

TABLE No. 7.

Color of Fat		Character of Pigments.
Yellow	Red	
6.0	1.0	Petroleum ether quant. extracted cholesterol-free pigment from 80 per cent alcohol. Solubility test showed carotin and xanthophylls.
2.5	0.7	Chromotogramm of xanthophylls showed adsorbed yellow constituent and unadsorbed orange zone.
1.4	0.5	Pigment from 240 gm. fat had color of 35 yellow, 1.0 red. Solubility test showed both carotin and xanthophylls, as did also absorption bands.

The Pigments of Butter Fat After Carrot Feeding.—The fat tested was taken during a carrot feeding experiment with the same cow used in the above experiments. This feeding experiment directly followed the third period of very light colored fat. The color of the fat was 28 yellow and 1.4 red. Solubility tests on the pigment showed carotin and a very small amount of xanthophyll. The petroleum ether soluble part of the pigment gave a CS_2 chromotogramm of a single unadsorbed rose colored zone. The filtered pigment showed three well defined absorption bands. I, 225-245; II, 263-283; III, 301-320.

The Pigments of the Fat from Colostrum Milk.—It is a well known fact that the first milk drawn after parturition always has a high yellow color. It is not generally known, however, that this high color is usually due [1] entirely to the suspended fat globules. We have many times observed, not only in connection with this study but also in connection with numerous studies dealing with the chemical composition of milk, that when the fat is entirely removed from colostrum milk the skim milk has the appearance of ordinary skim milk, and the butter and the rendered fat have a depth of color which is never equaled at any subsequent stage of the lactation period. This characteristic of colostrum milk is common to all breeds of cows, and the high color of the fat continues in cows of all breeds for a short time after parturition and then gradually falls off. Table 8 gives the color of the milk fat of several cows shortly after parturition and again a week or two later. The color readings are the Lovibond tintometer readings of a one-inch layer of melted, rendered fat.

TABLE NO. 8. COLOR OF THE FAT OF COLOSTRUM MILK.

Cow No.	Breed	Roughage fed.	Days after Parturition.	Color		
				Yellow	Red	Light
301	Ayrshire	Alfalfa(a)	4	78.0	3.5	1.0
301	Ayrshire	Alfalfa	26	71.0	1.5	0.5
300	Ayrshire	Alfalfa	4	71.0	3.5	1.0
300	Ayrshire	Alfalfa	20	68.0	2.8	1.0
2	Jersey	Alfalfa	13	68.0	2.6	0.5
2	Jersey	Alfalfa	22	57.0	2.5	0.5
2	Jersey	Alfalfa	2	54.0	4.3	1.0
2b	Jersey	Alfalfa	20	50.0	2.5	1.0
20	Jersey	Alfalfa	2	47.0	4.8	1.0
20	Jersey	Alfalfa	25	47.0	2.0	0.5
206	Holstein	Alfalfa	1	50.0	4.7	0.3
206	Holstein	Alfalfa	5	54.0	2.0	0.2

(a) The Alfalfa hay was rich in carotin and xanthophylls.
(b) Second sample taken after next parturition.

This phenomenon at once offered the interesting problem of the relation of the colostrum pigment to the pigment of normal butter. A close study was accordingly made of the pigment of the fat from the first milk drawn after parturition. The cow selected

1. Colostrum milk is occasionally contaminated with blood.

for study was a pure bred Holstein. The fat tested had a very high color; a one inch layer gave a reading of 64 yellow, 5.0 red and 1.0 light in the Lovibond tintometer.

The unsaponifiable pigment and impurities from fifteen grams of the fat had a golden yellow color in ether and in carbon bisulphide a blood red color. This solution was analysed chromotographically. The entire pigment passed through unadsorbed as a red orange or rose colored zone, leaving no adsorbed zones and no pigment behind in the $CaCO_3$ which could be washed out with ten per cent alcoholic petroleum ether. The filtered solution showed two strong absorption bands and a third faint one. (See Table 9.)

The pigment was now analyzed according to its proportionate solubility in petroleum ether (b. p. 30-50° C) and eighty per cent alcohol, and was thus divided into two portions, a major portion extracted by the petroleum ether and a very minor portion which the petroleum ether would not extract from the eighty per cent alcohol.

The carotin-like pigment thus obtained was freed from cholesterol by the digitonin method and its bands again measured in carbon bisulphide solution. (See Table 9.)

The residue from this solution gave a beautiful transient blue color with concentrated H_2SO_4 and a very transient blue-green color with concentrated HNO_3.

The eighty per cent alcohol soluble pigment showed three absorption bands in carbon bisulphide solution, the first two being a little more intense than the third. (See Table 9.) While in this solution the pigment was analyzed by means of a chromotogramm and showed two zones, a primary little adsorbed zone of orange color, and a secondary more adsorbed zone of yellow color. Hydrochloric acid gave no color reaction with the alcoholic solution of the pigment of either zone.

TABLE 9. ABSORPTION BANDS OF PIGMENTS OF COLOSTRUM MILK FAT.

	Combined Pigment (CS_2 Solution)	Carotin (CS_2 Solution)	Xanthophylls	
			(CS_2 Sol.)	(C_2H_5OH Sol.)
Band I	223–240	224–242	232–249	264-281
Band II	260–278	259–278	272–291	306–326
Band III	300–320	302–319	312–330	356–. . .

Crystalline Form of Carotin From Butter Fat.—The great concentration of pigment in the fat from colostrum milk seemed to offer an excellent opportunity to at least attempt the isolation of the pig-

ment in crystalline form. The pigment was isolated in the usual way from forty grams of very high colored colostrum fat from a Jersey cow. Great care was taken during the isolation to avoid aldehyde resin pigments. This was successful. The ether solution of the unsaponifiable substances was evaporated at 35° C. and the residue dissolved at once in carbon bisulphide. This solution, which had a blood red color, was concentrated to 2 c. cm. volume at a low temperature and an excess of cold absolute alcohol added. There was no immediate crystallization, but after standing several days there were deposited a number of yellow crystals. These were at first thought to be crystals of the pigment, for similar crystals were obtained in the same manner from the corpus luteum pigment. The crystals were perfectly formed double pyramidal forms, but proved to be crystals of sulphur which evidently arose from the carbon bisulphide used. A further attempt at crystallization of the pigment was abandoned. It undoubtedly would prove successful if sufficient pure material could be obtained.

THE RELATION BETWEEN THE COLOR OF THE MILK FAT AND THE FOOD OF THE COW.

General observation for no doubt hundreds of years, at least, ever since butter has become of importance in the diet of man, has shown that green feeds of all kinds, especially fresh green grass greatly increase the color of butter fat. Other feeds, such as carrots, beets and yellow corn have been said to have the same effect. It has never been the subject of a scientific investigation however, to show just what relation exists between the food of the cow and the color of the milk fat. With a chemical relation established between the milk fat pigments and carotin and xanthophylls, the relation of the color of the milk fat to the food seems to be readily explained on the ground that the foods that have been observed to cause the highest colored butter, namely, green foods, carrots, etc. are those which are especially rich in carotin and xanthophylls, particularly carotin, as in the case of the carrots. Indeed we can go still further and definitely state that these pigments must be abundantly present in the food before the milk fat will show a high color, as will be demonstrated by the experiments which are about to be reported.

Character of the Yellow Pigments of the Common Cattle Feeds.— The chemical study of the yellow pigment of milk fat, shows that its principal constituent belongs to the hydrocarbon or carotin group of pigments, although it also contains xanthophylls as a very minor secondary constituent. It accordingly became necessary to study the

nature of the pigments of various common cattle foods before conducting any feeding experiments to prove that there is a direct relation between the presence of carotin in the butter fat and the presence of carotin in the food. It was at first merely sought to show the presence or absence of unsaponifiable yellow pigments in various foods, by extracting them with hot alcohol, saponifying the extract with potassium hydroxide and extracting the pigment from the soap with ether. From this standpoint, it was found that among the grains and concentrates, wheat bran, linseed meal, dried brewer's grains and cottonseed meal all showed the presence of small amounts of unsaponifiable yellow pigments, while white corn was found to be the only common grain absolutely free from such pigments. Yellow corn was of course found to be rich in unsaponifiable yellow pigments. Some roughages such as corn silage and cottonseed hulls were found to contain small amounts of unsaponifiable pigments, while wheat straw and oat straw were practically free from them. The hays were found to be the most variable of all feeds. All green hays,[1] such as alfalfa, first-class clover and the very best timothy were found to contain considerable amounts of unsaponifiable yellow pigments, the amounts varying with the greenness of the hay. Bleached hays, whether timothy, clover, or alfalfa were more or less free from unsaponifiable yellow pigments.

Some feeds were investigated more particularly with a purpose of showing the character of the unsaponifiable yellow pigments. Without reporting the experimental details in all cases, but merely stating that the methods of analysis were spectroscopic, chromotographic, and solubility methods, the following results were obtained.

Cotton Seed Meal and Cotton Seed Hulls.—It was found that the unsaponifiable yellow pigments of cottonseed meal and cottonseed hulls were due entirely to the oil they contain; and further that this oil which is known to be characterized by its yellow color contains equal proportions of carotin and xanthophyll. The carotin is the usual one met in other places, while the xanthophyll is made up of at least five different constituents according to their adsorption properties. The chief xanthophyll is not adsorbed to any extent by $CaCO_3$ from its carbon bisulphide solution, and in this solvent shows absorption bands of characteristic position, shifted considerably toward the blue from the normal xanthophyll bands. In CS_2 the bands measured: I, 238-260; II, 285-303; III, 355—. The remainder of the xanthophylls were so firmly held by the $CaCO_3$ that they could not be readily

1. By green hay is meant hay that has been cured under such conditions that it still retains a large part of the green color which characterizes its uncut condition. The green alfalfa hay referred to throughout this paper was western cured alfalfa which had a remarkably bright green color.

washed out with a stream of carbon bisulphide. They were all washed out however, by a one per cent alcoholic petroleum ether solution.

Bleached Alfalfa Hay.— This hay was quite free from green stalks and as it had been found palatable to the cows it was of special interest as an experimental roughage for non-pigmented feeding studies. A selected sample of the hay was ground up fine, and extracted with ten per cent alcoholic petroleum ether. The light green colored extract showed no indication of either carotin or xanthophyll when analyzed by means of the chromotogramm, and no yellow pigment was extracted from its alcoholic solution by petroleum ether or by carbon bisulphide.

Yellow Corn.—The unsaponifiable yellow pigment of yellow corn is in all probability in the oil. It was found to be composed of two constituents, the largest part of which is a xanthophyll-like pigment, showing absorption bands in alcoholic and carbon bisulphide solutions lying close to the normal xanthophyll bands. In CS_2 the bands measured: I, 229-251; II, 274-291; III, not measured. It was not adsorbed from either petroleum ether or carbon bisulphide by $CaCO_3$ but passed through as a yellow or orange zone. Its carbon bisulphide solutions were orange colored. Petroleum ether readily extracted the pigment from its concentrated eighty per cent alcoholic solution, but it could be completely re-extracted from its petroleum ether solution by fresh eighty per cent alcohol. In this respect it differed from any xanthophyll-like pigment yet investigated. The pigment was more soluble in carbon bisulphide than in eighty per cent alcohol and in this respect favored carotin. On warming its alcoholic solution containing a little concentrated hydrochloric acid, the color of the solution changed to a light greenish blue, with the fading of the absorption bands. The minor constituent of the corn pigment had the spectroscopic, solubility and adsorption properties of carotin.

The Carrot.—It was planned to conduct some feeding experiments with carrots, and a special study of its relative proportion of carotin and xanthophyll was accordingly considered advisable. A large well-colored carrot was scraped, chopped fine, and boiled in water for one hour. The softened tissue was poured onto cheesecloth and the water squeezed out of the pulp. The pulp was pressed through a wire gauze, dried and powdered. The meal was extracted with carbon bisulphide and a portion of the blood red solution analyzed by means of a chromotogramm. By far the greatest part of the pigment passed through unadsorbed as a rose colored zone, leaving a small amount of adsorbed pigment in the column which was not differentiated into zones but which was readily washed out with alco-

holic petroleum ether. The proportion of xanthophyll to the entire pigment in this test was very small, not over three or four per cent.

The rose colored carotin solution showed three beautiful absorption bands, all of equal intensity. The measurements of the bands were completely in accord with the standards given in Table 6.

The remainder of the carbon bisulphide extract of the dried carrot meal was evaporated into alcohol. The alcohol was diluted with water to an eighty per cent solution and extracted with petroleum ether (b. p. 30°-50° C) until no more color was extracted. The alcoholic solution of the xanthophylls which remained was combined with the solution of the xanthophylls washed out of the chromotogramm with alcoholic petroleum ether. The combined solutions were evaporated to dryness and the residue dissolved in a small amount of carbon bisulphide. This solution showed three absorption bands and end absorption. The measurements were the same as given in Table No. 6.

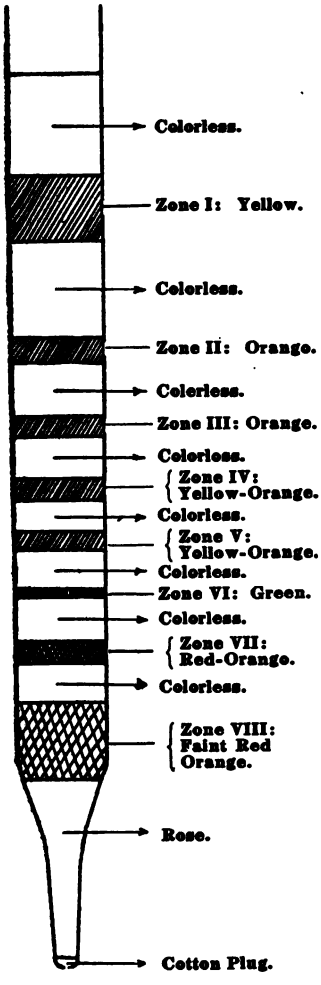

FIGURE IV.

This carbon bisulphide solution was orange colored when concentrated and gave the chromotogramm shown in figure IV. There was a small amount of carotin pigment present which being unadsorbed was readily washed out and caught at the lower end of the tube. Zone VIII was also only slightly adsorbed and was washed out by a stream of carbon bisulphide. When this zone was washed out the suction was stopped. The $CaCO_3$ tinted zones were then removed separately from the column with the aid of a knife; the CS_2 was drawn off in each case at a low temperature; and the pigment washed out with ninety-five per cent alcohol.

Zone II was found to contain the most pigment, judged from the color of its alcoholic solution, with the lower zones about the same with the exception of Zone V which had very little pigment. Zone I contained considerably more pigment than was indicated by the color of its zone in the chromotogramm. The spectroscopic properties of all the xanthophyll constituents were studied with respect to the normal xanthophyll bands. The results

are shown in Table 10. The observations were not made by observing all the pigments in the same volume but the volumes were adjusted in each case to give the best possible bands.

It will be noticed that with the exception of Zone I, all the xanthophylls showed the three normal xanthophyll bands to some degree of intensity. Similarly hydrochloric acid had no effect on any of these solutions or on the bands. Nitric acid had the same effect on the pigments of Zones II, VII, and VIII causing the solutions to fade with the disappearance of the bands. The effect of nitric acid on the pigments of Zones III and IV was somewhat different. In these two cases a fourth well-developed band appeared before the solution lost its color and the three normal bands faded. The pigment of Zone I was entirely different from any of the others. The normal first band of the xanthophylls was entirely missing and the alcoholic solution turned a distinct bluish green on the addition of a little concentrated hydrochloric acid. This color persisted for 24 hours, long after the absorption bands had disappeared. This was evidently the xanthophyll *B* of Tswett and the xanthophyll *B* of C. A. Schunck.

TABLE NO. 10. SPECTROSCOPIC PROPERTIES OF THE XANTHOPHYLLS OF THE CARROT.

Zone	Band I	Band II	Band III	End Absorption
I	Missing	Good	Good	Very faint
II	Fair	Fair	Fair	None
III	Very strong	Very strong	Weak	None
IV	Strong	Very strong	Faint	None
V	Very faint	Good	Good	None
VII	Very strong	Very strong	Very faint	None
VIII	Good	Good	Very faint	Very faint

THE FEEDING EXPERIMENTS.

The foregoing studies indicated that the foods best adapted for non-pigmented rations were bleached hays and cottonseed hulls for roughages and white corn and cottonseed meal for grains. To study the variation in the color of butter fat, the ration of various cows was changed to one containing the smallest possible amounts of carotin and xanthophylls, and the butter fat studied colorimetrically during this time. The procedure for the butter fat was in each case as follows: The milk of one or two milkings was separated by means of a centrifugal hand separator and the cream churned by hand in

quart bottles. The butter thus obtained was rendered at a temperature of 50° to 60° C. and the rendered fat filtered. The pure filtered fat was analyzed colorimetrically by means of the Lovibond tintometer and its standard color glasses. The color of the fat was always compared in one-inch layer. The Lovibond tintometer is shown in Figure V.

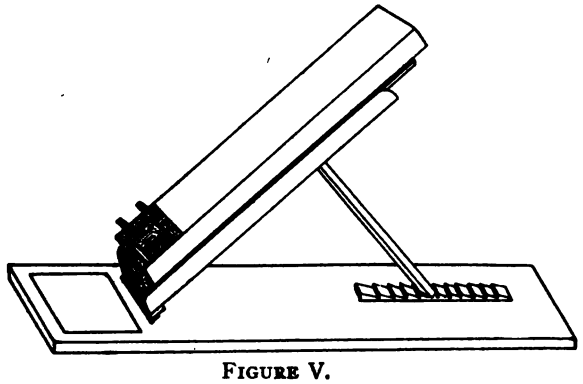

FIGURE V.

The solution (in the present case melted butter fat) whose color is to be measured is placed in a cell with glass ends (one inch apart in all this work), and the color matched by standard color glasses of various units of yellow, red or blue, and the color of the solution read by adding together the various glasses of color used to match the unknown color. Melted butter fat having an orange tint requires only yellow and red to match its color. All readings are made with the instrument pointing towards the daylight (not sunlight). The instrument is quite sensitive towards the yellow glasses below 25 units of yellow but the sensitiveness decreases considerably above 40 units of yellow. In other words, it is possible to match the exact color of an "unknown" much more closely when its color is below 30 to 35 units of yellow than when its color is above this value. In a great many cases, and this nearly always applies to butter fat, it is possible to match the tint of the fat but the color of the fat is more brilliant than that of the combined standard glasses. In this case an exact match can be obtained by "damping down" the butter fat color by inserting in front of it equal units of the three colors, yellow, red and blue, and recording this as "light."

Before reporting the data dealing with the variation in the color of the butter fat it may be possible to convey some idea of what the various colors mean when applied to butter fat by stating that rendered "June" butter in the one-inch cell will give a color of from 80 to 60 units of yellow. Color readings between 45 and 25 units of

yellow would accordingly indicate a fairly well colored to light colored butter, between 20 and 8 units of yellow would be called light to very light colored butter, while below these limits ranging down to 1 or 2 units of yellow would be called white to "dead" white, especially if the fat was still in the form of butter.

Experiment 1.

The ration of Cow No. 57, a pure bred Jersey, was changed from a ration rich in carotin and xanthophylls to a ration containing a very small amount of these pigments. The ration rich in carotin and xanthophylls consisted of alfalfa hay and yellow corn. The ration poor in these pigments was composed of bleached clover hay and white corn. The results of the experiment are shown in Table 11.

The change from a ration rich in carotin and xanthophylls to one poor in these pigments caused the color of the butter fat to drop from 43 units of yellow to 8.5 units of yellow, from a well colored to a very light colored fat. This change of color was very gradual and required 29 days. It should be stated, however, that the cow did not relish her non-pigmented ration. She lost weight regularly, and her milk production fell off a great deal. It was apparent that the animal was drawing heavily during this entire period from a storage of pigment in her body. It will be shown in the subsequent papers of this series that in this experiment the blood and also the body fat were supplying the pigments for the milk fat.

It may be stated that a slow lowering of the color of the milk fat, such as took place in this experiment, would be normal for all Jersey cows whose ration is changed to an unpalatable, non-pigmented one like that used in this experiment. The explanation for this is found in the high color of the body fat of this breed of cows. We therefore have here a clear explanation of why Jersey cows will sometimes apparently give yellow milk fat during the winter months when their food is almost or entirely lacking in carotin and xanthophylls. Under these conditions if the body fat is called upon to supplement the digestion products of the food in the production of milk fat at the same time the blood serum storage of pigments is being drawn upon, it is clear that the reduction in color of the milk fat will be very gradual, as in the case of Cow No. 57, and a complete elimination of color may require a long period of time.

Continuing the discussion of the experiment it is seen that when the color of the milk fat had dropped to 8.5 units of yellow the white corn in the ration was replaced by mixed corn, white and yellow

(mostly yellow). Later this was replaced by yellow corn entirely. The roughage remained the same, i. e. bleached clover hay. The table shows that yellow corn had no effect whatever upon the color of the milk fat. There seemed to be a very slight effect at first but the color of the fat soon dropped back to 8.0 units of yellow.

TABLE No. 10. THE EFFECT OF A CAROTIN AND XANTHOPHYLL-FREE RATION ON THE COLOR OF MILK FAT. JERSEY COW No. 57.

Date	Pounds hay per day	Pounds grain fed per day		Color of butter fat.		
				Yellow	Red	Light
1912		Corn	Mixture			
Mar. 1	15(a)		8	43.0	2.0	0.2
2	15(b)		8			
3	15	4 white	4			
4	15	4 white	4			
5	15	4 white	4			
6	15	8 white				
7	15	8 white		33.0	2.0	0.2
8	15	8 white				
9	15	8 white		29.0	1.7	0.2
10	15	8 white				
11	15	8 white		33.0	1.8	0.2
12	15	8 white		26.0	1.6	0.2
13	15	8 white		26.0	1.7	0.2
14	15	8 white		22.0	2.0	0.2
15	15	8 white		22.0	2.0	0.2
16	15	8 white		21.0	1.8	0.2
17	15	8 white				
18	15	8 white		18.0	1.7	0.2
19	15	8 white		19.0	1.6	0.2
20	15	8 white		18.0	1.6	0.2
21	15	8 white				
22	15	8 white		12.0	1.6	0.2
23	15	8 white				
24	7	10 white				
25	7	10 white		11.0	1.6	0.2
26	7	10 white		11.0	1.6	0.2
27	7	10 white		10.0	1.5	0.1
28	7	10 white		10.0	1.5	0.1
29	7	10 white		8.5	1.8	0.2
30	7	10 white		9.0	1.7	0.2
31	7	10 mixed		10.0	1.8	0.2

(a) Alfalfa.
(b) Clover—Mar. 2 to Mar. 31.

The 7 lbs. of clover hay was now replaced by 10 lbs. of alfalfa hay rich in carotin and xanthophylls. The effect on the color of the

TABLE 11 (CONTINUED). EFFECT OF FEEDING YELLOW CORN TO A COW GIVING LOW COLORED MILK FAT. JERSEY COW NO. 57.

Date	Pounds hay per day	Pounds grain fed per day	Color of butter fat		
			Yellow	Red	Light
1912		Corn			
Apr. 1	7(a)	10 mixed	8.5	1.7	0.2
2	7	10 mixed	8.0	2.1	0.2
3	7	10 mixed	8.0	1.9	0.2
4	7	10 mixed	12.0	1.5	0.2
5	7	10 mixed	12.0	1.7	0.2
6	7	10 mixed	11.0	1.8	0.2
7	7	10 yellow			
8	7	10 yellow	11.0	1.8	0.2
9	7	10 yellow	8.0	1.7	0.2
10	7	10 yellow	9.0	1.7	0.2
11	10(b)	10 yellow	10.0	1.5	0.2
12	10	10 yellow	12.0	1.6	0.2
13	10	10 yellow	15.0	1.6	0.2
14	10	10 yellow	20.0	1.6	0.2
15	10	10 yellow	33.0	1.7	0.2
16	10	10 yellow	36.0	1.6	0.2
17	10	10 yellow	38.0	1.6	0.2
18	10	10 yellow	43.0	1.6	0.2
19	10	10 yellow	45.0	2.0	0.5
21	10	10 yellow	43.0	1.8	0.5
22	10	10 yellow	43.0	1.7	0.5
23	10(c)	10 yellow	46.0	1.7	0.5
24	10	10 yellow	40.0	1.8	0.5
25	10	10 yellow	43.0	1.8	0.5
27	10	10 yellow	52.0	2.0	0.5
30	10	10 yellow	57.0	2.1	0.5
May 2	10	10 yellow	64.0	2.1	0.5
4	10	10 yellow	64.0	2.2	0.5
5-10(d)	Pasture only		80.0	2.5	0.5

(a) Clover—Apr. 1-10.
(b) Alfalfa—Apr. 11-22.
(c) Alfalfa and pasture—Apr. 23-May 10.
(d) Sample taken May 10.

milk fat was immediate. At the end of seven days the color had increased to 43 units of yellow, the maximum supplied by this roughage. Later the ration was supplemented by some fresh pasture grass. The color of the milk fat increased to 64 units of yellow. Still later the cow was turned out to pasture alone. The color then reached the maximum we have observed, i. e. 80 units of yellow.

Experiment No. 2.

This experiment was conducted with Cow No. 301, a pure bred Ayrshire. A short time previous to the experiment here reported this

cow was in very poor flesh resulting from a feeding experiment in which she was heavily underfed. Her ration during her underfeeding was composed of alfalfa hay rich in carotin and xanthophylls and a grain mixture of corn, bran and linseed meal. At the end of the underfeeding experiment the ration was changed to bleached alfalfa hay, the pigmentation of which was reported above, and white corn and cottonseed meal. Enough of this ration was given to bring the cow back to normal feeding conditions. At the end of this time the experiment here reported was begun. The results are given in Table 12.

At the end of ten days the color of the milk fat had dropped to 9 units of yellow. When it was apparent after a week's further trial that the color had reached at least a temporary minimum value for this ration, the grain was changed to yellow corn entirely. This was finally increased to 12 lbs. per day. The result was very clearly in accord with that of Experiment No. 1, showing that yellow corn is not a source of pigment for the milk fat of dairy cows.

TABLE No. 12. EFFECT OF NON-PIGMENTED RATION AND A RATION CONTAINING YELLOW CORN UPON THE COLOR OF MILK FAT. AYRSHIRE COW No. 301.

Date of sample	Pounds alfalfa hay	Pounds corn	Pounds cotton-seed meal	Color of butter fat	
				Yellow	Red
1912					
October 3	16	6(a)	2	27.0	1.7
13	16	6	2	9.0	1.7
17	16	6	2	6.0	1.5
21	16	6	2	9.0	1.2
24	16	8(b)		7.5	1.2
25	16	8		9.0	1.5
26	16	8		10.0	1.5
27	16	8		9.0	1.5
28	16	8		9.0	1.5
29	16	8		10.0	1.5
30	16	8		8.5	1.2
31	16	8		10.0	1.2
November 1	16	12		9.5	1.2
2	16	12		10.0	1.2
3	16	12		8.5	1.2
4	16	12		8.0	1.2

(a) Oct. 3-21, white corn.
(b) Oct. 24-Nov. 4, yellow corn.

Experiment No. 3.

This feeding experiment was conducted with the same cow as the preceding experiment and immediately followed that experiment.

It seemed very probable that the reason the color of the milk fat in Experiments 1 and 2 could not be lowered more than the uniform figure found in both experiments, i. e., about 8 units of yellow, was due to the fact that the ration was supplying a small amount of pigment and also to the fact that the normal storage, that in the blood serum, had not been exhausted. It seemed reasonable to suppose, therefore, that if the first factor was eliminated at the outset, the second would necessarily also be eliminated if the experiment was continued for a sufficient length of time. The experiment here reported was for the purpose of testing the validity of this supposition, and also for the purpose of ascertaining to how low a point the color of the milk fat could be reduced. The ration chosen was one which would supply the least amount of carotin and xanthophylls. It was composed of cottonseed meal and cottonseed hulls. The results of the experiment are given in Table 13.

The supposition stated above was fully borne out by the long continued feeding of a practically non-pigmented ration. At the end of 52 days' feeding, the cow was producing absolutely colorless butter. It was only when the rendered butter was viewed in the tintometer that a very slight amount of color could be detected. This very slight amount of color was due to the fact that the normal storage of pigment in the body, that in the blood serum, had not been completely exhausted, as will be shown in a subsequent paper of this series. It is probable also that the body was being drawn upon for some of its pigments, for the animal suffered somewhat from underfeeding on this ration. It is also possible that a very small amount of carotin was being supplied by the oil in the cottonseed meal and hulls. For practical purposes such a supply of pigment would of course be considered absolutely negative.

TABLE NO. 13. EFFECT OF A LONG-CONTINUED FEEDING OF A NON-PIGMENTED RATION UPON THE COLOR OF MILK FAT. AYRSHIRE COW NO. 301.

Date of sample	Pounds alfalfa hay	Pounds cotton-seed hulls	Pounds cotton-seed meal	Pounds Corn	Color of butter fat	
					Yellow	Red
1912-13						
Nov. 5	6.5			12	9.0	1.2
6	4.5	0.5	0.5	10	8.0	1.2
7	8.0	1.0	1.0	8	9.0	1.1
8	8.0	2.0	2.0	7	8.0	1.0
9	8.0	2.0	2.0	6	7.0	1.0
10	7.0	3.0	3.0	6	6.5	1.0
11	6.0	3.0	3.0	5	6.0	1.0
12	6.0	4.0	4.0	4	5.5	0.9
13	6.0	4.0	4.0	4	5.0	0.8
14	3.0	5.0	5.0	2	5.0	0.8
15	2.0	6.0	6.0		5.0	0.9
16		10.0	6.0		4.0	0.7
17		12.0	6.0		4.5	0.8
18		12.0	7.0		4.0	0.7
19		12.0	7.0		3.5	0.7
20		12.0	8.0		3.5	0.7
21		13.0	8.0		3.5	0.7
22		14.0	8.0		3.5	0.7
23		14.0	8.0		3.2	0.7
24		16.0	8.0		3.1	0.7
25		16.0	8.0		3.0	0.7
26		16.0	8.0		2.6	0.5
27		16.0	8.0		3.2	0.7
28		16.0	8.0		2.5	0.7
29		16.0	8.0		3.0	0.7
Jan. 2		16.0	8.0		1.4	0.5
7		16.0	8.0		1.3	0.4

It will be of interest to state in connection with this experiment that it furnished the three "light colored" fats for the studies of the proportion of carotin and xanthophyll which were reported above.

Experiment 4.

The color of the butter fat of Cow No. 301 was now so low that the conditions were considered ideal for one or two additional important investigations, first a confirmation of the apparently negative effect of feeding yellow corn which was obtained in Experiments 1 and 2, and second a study of carrot feeding. In the latter study the results would be especially interesting in view of the fact that the pigment fed would be almost pure carotin. The hay used in this experiment was a very light colored timothy hay which was not quite so free from carotin and xanthophylls as the bleached alfalfa of Experiment 3, but which apparently had no effect on the color of the fat

as the data will show. The results of the two studies are given in Table 14.

In regard to yellow corn feeding, it is seen that replacing 2 lbs. cottonseed meal and 4 lbs. white corn by 6 lbs. yellow corn had no influence upon the color of the milk fat. If yellow corn has any value as an aid in the production of yellow milk fat, it would certainly have been evident in this experiment where the milk fat was practically colorless before feeding the yellow corn. This experiment therefore gives conclusive proof of the negative value of yellow corn in the pigmentation of milk fat. The reason for this will be discussed more fully in the general discussion of the experiments.

TABLE NO. 14. EFFECT OF A RATION CONTAINING YELLOW CORN AND OF A RATION CONTAINING CARROTS UPON THE COLOR OF MILK FAT. AYRSHIRE COW NO. 301.

Date of sample		Pounds roughage(a)	Pounds carrots	Pounds cottonseed meal	Pounds corn	Color of butter fat	
						Yellow	Red
1912							
Jan.	3	14		4	4 white		
	21	12		4	4 white		
	24	12		4	4 white	1.2	0.4
	25	12		4	4 white		
	28	12		2	6 yellow		
	29	12		2	6 yellow	1.4	0.5
	30	12		2	6 yellow	1.5	0.4
	31	12		2	6 yellow	1.7	0.4
Feb.	1	12		2	6 yellow	1.7	0.4
	2	12		2	6 yellow	2.0	0.5
	3	12		2	6 yellow	1.8	0.5
	4	12		2	6 yellow	2.0	0.5
	5	12		2	6 yellow	1.8	0.3
	6	12		2	6 yellow	2.0	0.5
	7	12	6	4	4 white	2.2	0.5
	8	12	20	4	4 white	2.2	0.5
	9	12	30	4	4 white	2.3	0.5
	10	12	40	4	4 white	3.2	0.6
	11	12	40	4	4 white	4.5	0.7
	12	12	50	4	4 white	5.5	1.0
	13	12	50	4	4 white	8.0	1.0
	14	12	50	4	4 white	11.5	1.0
	15	12	30	4	4 white	16.0	1.1
	16	12	30	4	4 white	15.0	1.2
	17	12	20	4	4 white	18.0	1.3
	18		20			19.0	1.1
	19	3				15.0	1.1
	20		5			10.0	1.3
	21	3	10	1	1 white	8.0	1.2
	22	3	10	2	2 white	9.0	1.3

(a) Roughage consisted of 2 parts bleached timothy hay and 1 part cottonseed hulls.

TABLE No. 14. (CONTINUED)

Date of sample		Pounds roughage(a)	Pounds carrots	Pounds cottonseed meal	Pounds corn	Color of butter fat	
						Yellow	Red
1913							
Feb.	23	12	20	4	4 white	11.0	1.3
	24	12	20	4	4 white	21.0	1.3
	25	12	20	4	4 white	28.8	1.2
	26	12	20	4	4 white	36.0	1.8
	27	12	20	4	4 white	27.0	1.3
	28	12	20	4	4 white	24.0	1.3
Mar.	1	12	20	4	4 white	24.0	1.3
	2	12	20	4	4 white	28.0	1.4
	3	12	20	4	4 white	26.0	1.4
	4	12	20	4	4 white	26.0	1.3
	5	12	20	4	4 white	24.0	1.4
	6	12	50	4	4 white	24.0	1.3
	7	12		4	4 white	23.0	1.3
	8	12		4	4 white	19.0	1.2
	9	12		4	4 white	19.0	1.2
	10	12		4	4 white	18.0	1.6
	11	12		4	4 white	17.0	1.1
	12	12		4	4 white	14.0	1.1
	13	14		4	4 white	11.0	1.1
	14	14		4	4 white	12.0	1.2
	15	14		4	4 white	12.0	1.2
	17	14		4	4 white	10.5	1.2
	21	14		4	4 white	7.5	1.2
	24	12		4	4 white	7.0	1.0
	30	12		4	4 white	7.5	1.3

(a) Roughage consisted of 2 parts timothy hay and 1 part cottonseed hulls.

Note: During the period from 2:18 p. m. to 2:23 a. m. the cow was badly off feed and her entire ration was withdrawn for a few days. She soon recovered, however, and was put back on the experimental ration, not feeding so many carrots, however.

In regard to the effect of feeding carrots, the data brings out several interesting points. The carrots were added to the ration on February 7, and the amount was rapidly increased to 50 lbs. per day. The cow ate them with a good deal of relish for about a week when she began to refuse part of them and finally went off feed entirely, making it necessary to withdraw her entire ration for a day or two. The effect of this carrot feeding period upon the color of the milk fat was to increase the color from 1.8 to 19 units of yellow. This increase was not nearly as great, however, as was expected considering the large amount of carrots that was fed and the length of time they had been in the ration, i. e. eleven days.

It is clear from a study of the subsequent data that this abnormal result was due to some physiological disturbance that finally resulted in the cow going entirely off feed. As soon as the carrots were removed from the ration, the color of the fat dropped at once to 8 units of yellow. However, when the animal had recovered from her attack of indigestion, and the carrots were again added to the ration, the effect upon the color of the milk fat was perfectly normal. On the fourth day of feeding 20 lbs. of carrots the color had reached a maximum of 36 units of yellow. The color later dropped a little with an increase in fat production which is not shown in the table, but remained in the neighborhood of 28 units of yellow until the carrots were removed. The color then began to drop slowly in the normal way. After dropping to 7 units of yellow on the twenty-third day after the carrots had been removed, the experiment was stopped.

This experiment furnished the samples of butter fat whose proportion of carotin and xanthophyll were studied and reported in an earlier part of this paper, namely the fat after carrot feeding.

Experiment 5.

This was a second carrot feeding experiment using another cow, i. e. Cow No. 221, a pure bred Holstein cow. The experiment was not as successful as was hoped because of the peculiar appetite of the cow. She refused to eat more than ten pounds of the carrots per day except on two days so the experiment was discontinued. The data are given in Table 15. Notwithstanding the peculiar appetite of the cow it is interesting to note that the feeding of only 10 lbs. of carrots per day for 8 days was sufficient to bring the color of the milk fat almost back to the starting point, i. e. 26 units of yellow.

TABLE No. 15. EFFECT OF A NON-PIGMENTED RATION AND OF A RATION CONTAINING CARROTS UPON THE COLOR OF MILK FAT. HOLSTEIN COW No. 221.

Date of sample	Pounds hay(a)	Pounds carrots	Pounds cotton-seed meal	Pounds corn	Color of butter fat	
					Yellow	Red
1912 Nov. 29	Normal herd ration containing green alfalfa hay.				26.0	1.5
Dec. 9	16		4	4	8.0	1.4
12	16		4	4	7.0	1.3
14	16		4	4	6.0	1.1
17	16	10	4	4	6.0	1.3
18	16	10	4	4	7.0	1.5
19	16	10	4	4	9.0	1.5
20	16	10	4	4	8.0	1.5
21	16	10	4	4	8.0	1.5
22	16	10	4	4	10.0	1.5
23	16	10	4	4	17.0	1.3
24	16	30	4	4	17.0	1.2
(a. m.) 25	16	20	4	4	14.0	1.5
(p. m.) 25	16	0	4	4	20.0	1.5
(a. m.) 26	16	0	4	4	18.0	1.5
(p. m.) 26	16	0	4	4	17.0	1.5
27	16	0	4	4	18.0	1.5
28	16	0	4	4	12.0	1.5
1913 Jan. 3	16	0	4	4	7.5	1.5
22	Herd ration since 1-4-13				14.0	1.2

(a) The hay was a mixture of equal parts of light colored timothy and bleached alfalfa.

Experiment No. 6

This was a feeding experiment the results of which were expected to corroborate those of Experiment 1, and show that the color of the milk fat of Jersey cows is as much dependent on the food as those of other breeds. The variation in the feed and the resulting color of the milk fat is shown in Table 16. A pure bred Jersey cow, No. 59, was used for this experiment.

TABLE NO. 16. EFFECT OF A NON-PIGMENTED RATION UPON THE COLOR OF MILK FAT. JERSEY COW NO. 59.

Date of feeding	Pounds grain mixture(a)	Pounds corn silage	Pounds hay	Date of sample	Color of butter fat		
					Yellow	Red	Light
1911							
March 11 to April 2	11.6	0	14(b)	April 2	46.0	1.8	0.5
April 3 to April 4	11.	10	9(c)	April 14	6.0	1.5	0.2
April 15 to April 23	11.	10	8	April 23	4.0	1.4	0.2
April 24 to May 8	11.	10	4	May 9	3.0	1.5	0.2
May 10 to May 20	11	10	4	May 20	3.0	1.5	0.2
May 21 to June 14	11.5	Corn Stover 5	8(d)	June 14	47.0	1.8	0.5
June 15 to June 29	12.	5	8	June 29	26.0	2.0	0.2

(a) The grain mixture was 5 lbs. corn and 6 lbs. cottonseed meal.
(b) Green alfalfa hay from March 11 to April 2.
(c) Bleached timothy hay from April 3 to May 20.
(d) Green alfalfa hay beginning May 21.

The results of this experiment show conclusively that Jersey cows are as much dependent upon their food for the pigments of the milk fat as other breeds of cows. In addition the experiment offers excellent proof of some statements previously made in explanation of the gradual lowering of the color of the milk fat of Cow No. 57 in Experiment No. 1. It was stated there that the yellow body fat was supplementing the normal storage of pigment on account of the unpalatableness of the ration. In the present experiment we have an example of the effect of changing the ration to a non-pigmented one

without causing the animal to draw upon any storage of pigment other than the normal one of the blood serum. The result was that the color of the milk fat dropped from 46 units of yellow to 6 units of yellow in twelve days, whereas in Experiment 1 it required thirty days to bring about a similar change of color. The very low color of the fat which was reached in Experiment 6 also indicates that only the normal storage was being drawn upon. Experiment 6 also shows that corn silage is not a source of pigment for the milk fat. The chemical changes which take place in this roughage evidently also largely destroy the carotin and xanthophylls. The chemical studies of the pigments of corn silage, which were reported above, showed this to be the case.

RELATION BETWEEN COLOR OF MILK FAT AND BREED OF COW.

The foregoing experiments have shown conclusively that dairy cows, exclusive of breed, are dependent on the carotin and xanthophylls in their feed for the pigment of their milk fat, in other words, that they cannot produce the pigment which is thus secreted. The question is at once raised as to wherein lies the so-called breed characteristic which is so much emphasized by the breeders of Guernsey and Jersey cattle? It will not be denied that a breed characteristic does exist in connection with the color of butter fat. We believe, however, that the data now to be presented will show that this breed characteristic has been overemphasized.

Since the butter fat is dependent upon the food of the cow for its color, it was necessary to compare the color of the butter fat of the different breeds under comparative feeding conditions, in order to obtain a correct estimate of the breed relation.

It would naturally be expected that the most favorable condition for studying the accuracy of the views held by the cattle breeders and others that some breeds of cows, such as the Jersey and Guernsey, are color producers while other breeds, such as the Holstein, are not color producers would be a comparison of the color of the combined fat of several cows of each breed. Table 17, which follows, gives such a comparison taken from animals in one herd. The milk and fat production of the various cows varied widely. The comparison was made during the winter months, the only source of pigment being a more or less variable quantity of green alfalfa hay in the ration, which was, however, the same for all the animals.

TABLE No. 17. RELATION OF BREED TO COLOR OF MILK FAT.

Breed	Color of butter fat		
	Yellow	Red	Light
Jersey	50.0	2.1	0.2
Ayrshire	38.0	1.7	0.2
Shorthorn	34.0	1.6	0.2
Holstein	31.0	1.7	0.2

The most striking fact brought out by this table is that the question of the color of the fat produced by the four breeds represented is not one of presence or absence of color, but rather a question of relative color. The fat from the Jersey cows was unquestionably the highest colored of the four samples but the fat from the Holsteins also had a very good color, although the butter would probably have been scored as "slightly low in color."

This point of relative color production is also clearly shown when comparing the fat produced by individual members of the breeds. Table 18 shows the color of the fat from two Jerseys and one Holstein cow under feeding conditions most favorable for the maximum color. These animals were producing about the same amount of butter fat, and the roughage of their ration consisted for the most part of freshly-cut soybeans, very rich in carotin and xanthophylls.

TABLE No. 18. SHOWING RELATIVE COLOR PRODUCTION BY DIFFERENT INDIVIDUALS.

Cow No.	Breed	Feed	Color		
			Yellow	Red	Light
59	Jersey	Fresh green soybeans and grain	54.0	2.5	1.0
34	Jersey	Ditto	60.0	2.5	1.0
208	Holstein	Ditto	29.0	1,8	0.5

When comparing the color of the fat produced by individual members of the Jersey and Holstein breeds under feeding conditions favorable for only a moderate amount of color in the fat, the relative color production of the breeds very nearly approaches unity. This is

especially true when the fat production and the actual proportion of the ration furnishing the pigments are taken into account. Such a comparison is shown in Table 19.

TABLE No. 19. RELATIVE COLOR PRODUCTION UNDER SPECIAL FEEDING CONDITIONS.

Cow No.	Breed	Pounds green alfalfa hay	Green feed (Dry matter basis) (a)	Pounds milk fat per day	Color	
					Yellow	Red
34	Jersey	10.0	42%	1.34	29.0	1.6
41	Jersey	12.0	41%	1.78	29.0	1.6
11	Jersey	8.0	40%	1.51	24.0	1.3
13	Jersey	8.0	45.5%	0.58	33.0	1.6
208	Holstein	15.0	35%	1.23	19.0	1.6
211	Holstein	12.0	28%	2.13	17.0	1.5
219	Holstein	12.0	28%	1.50	19.0	1.7

(a) Per cent of moisture free green feed in total ration.

The most interesting feature of the above table is to note that on the basis of the per cent of green dry matter in the ration, the Jersey cows produced the highest colored fat because they received the highest per cent of green dry matter. By taking into consideration also the difference in fat production, an interesting calculation can be made with the figures of average color, fat production and per cent of green dry matter in the ration, which will cause the relative color production of the two breeds to approach almost unity.

	Jerseys	Holsteins
Average per cent of green feed in ration	42.1%	30%
Average fat production	1.30 lbs.	1.6 lbs
Average color of fat (Units of yellow)	29.0	18.0

If it be assumed for the moment that there is no breed characteristic we can say that 30% green feed in the ration of the Holsteins produces 18 units of yellow in the fat for the same reason that 42% green feed in the ration of the Jerseys produces 29 units of yellow in their fat. If this is true then the following proportion would be a true one, i. e.:

$$42:29 :: 30:18$$

The product of the means is not quite equal to the product of the extremes but gives the result,

$$870 = 756$$

If the amount of fat produced is taken into consideration and each side of this equation is multiplied by the corresponding amount of fat we have the result,

$$870 \times 1.3 = 756 \times 1.6$$
$$\text{or } 1{,}131 = 1{,}210$$
$$\text{or } 1 = 1.07, \text{ which is very near unity.}$$

The relation between the breed of the cow and the color of the fat under two different conditions of feeding is well illustrated by Tables 18 and 19. The color of the fat produced by cows No. 34 and 208 is given under both heavy and moderate pigment feeding. The data in Table 19 were obtained a number of weeks after that in Table 18. The figures show that the change from heavy to moderate pigment feeding caused the color of the milk fat of the Jersey cow to drop 50% while a similar change in the feed of the Holstein cow caused a color drop of only 35%.

The relation of the breed to the change in color produced by a change in the ration is also well illustrated in the following table No. 20.

TABLE NO. 20. COLOR PRODUCTION IN DIFFERENT BREEDS AS AFFECTED BY CHANGES IN RATION.

Cow No.	Breed	Date	Grams fat produced	Color of fat	
				Yellow	Red
213	Holstein	3-11-13	122	8.5	1.4
213	Holstein	4-10-13	135	54.0	1.8
220	Holstein	3-11-13	167	3.0	0.7
220	Holstein	4-10-13	208	22.0	1.2
303	Ayrshire	3-11-13	213	2.5	0.6
303	Ayrshire	4-10-13	263	16.0	1.1
16	Jersey	3-11-13	304	11.0	1.7
16	Jersey	4-10-13	363	64.0	2.0
57	Jersey	3-11-13	240	5.2	1.2
57	Jersey	4-10-13	263	54.0	1.7
64	Jersey	3-11-13	281	4.7	1.5
64	Jersey	4-10-13	358	47.0	1.6

The first sample for each cow in the above table represents the result of a long continued feeding of a ration almost entirely lacking in carotin and xanthophylls. The second sample represents one month's feeding of a ration rich in these pigments, the ration including a

plentiful supply of green alfalfa hay and some fresh green grass. The data bring out two points worthy of emphasis. One of these is that it is possible to find pure bred Holstein cows entirely lacking in the so-called breed characteristic of color production. Holstein Cow No. 213 for instance produced as much color in her fat in both periods as any of the Jerseys. The low color of the milk fat of cows No. 220 and No. 303 in the second period can only be explained at present on the ground that it was due to some inherent characteristic of the animals, which for lack of a better term may be called breed characteristic. The other point brought out by the data is merely in emphasis of the results obtained in the feeding experiments showing that all breeds of cows suffer alike in regard to the color of their milk fat when the pigments carotin and xanthophylls are withdrawn from their food. At this time there is no breed characteristic.

Not only does the breed characteristic disappear when the source of the pigment is withdrawn, but it also disappears for all cows at the time of maximum color in the fat, i. e. immediately after parturition. Data was given in Table 8 showing the high color of the colostrum milk fat for cows of three breeds. There was certainly no breed characteristic evident there.

There is one other breed difference yet to be considered, which has led, probably more than anything else, to the belief that Jersey and Guernsey cows can produce yellow butter fat at any time, regardless of feed. This difference has to do primarily with the storage of pigment in the body, and its discussion belongs properly to the two subsequent papers of this series. A brief statement here in regard to it however will prevent a doubt arising in the minds of some readers, whose practical experience is apparently contrary to the experimental evidence here offered.

Stating the question in hypothetical form, it may be said that if a Jersey (or Guernsey) and a Holstein cow, both giving well-colored milk fat, the possibility of which cannot be denied in the light of the evidence which has been offered on this point, are put upon dry feed containing little or no carotin and xanthophylls, the color of the milk fat will drop much faster with the Holstein cow than with the Jersey (or Guernsey) cow, unless great care is taken to provide a ration as nourishing and palatable as the previous pigmented one. The result will be that the Jersey (or Guernsey) cow will appear to be producing colored milk fat on a non-pigmented ration. The explanation for this has already been given in connection with feeding Experiments Nos. 1 and 6, and lies in the fact that the body fat of Jersey and Guernsey cows furnishes a supplementary storage of pigments

not usually found in other breeds. It will be shown in a subsequent paper that if the body fat which furnishes the supplementary pigments in the case of the Jersey (or Guernsey) cow is laid on with a non-pigmented ration, it will be as colorless as is often seen in the case of the body fat of Holstein cows. If this were true in the hypothetical case described above, there would have been no breed characteristic evident, for it will also be shown in a subsequent paper that the normal storage of pigment, i. e. that of the blood serum, is practically the same for all breeds of cows.

Sufficient evidence has been presented to permit a repetition of a previous statement, namely that the relation of the breed to the color of the milk fat has received more emphasis than a study of the question will warrant. The color of the milk fat is primarily dependent upon the character of the food and the fact that some breeds of cows give less color in their milk fat than other breeds will probably be found to be only an apparent one when all the factors which come into play are known.

DISCUSSION OF RESULTS.

It was the primary object of this investigation to classify the natural yellow pigment of milk fat both as an individual and also in relation to the two well-known yellow classes of plant pigments, cartin and xanthophylls, whose general properties have often been observed to be closely related to various yellow pigments of so-called animal origin.

Basing the study upon a number of well-defined, characteristic physical and chemical properties of carotin and xanthophylls, it has been shown that the principal pigment of milk fat is a member of the fast widening group of hydrocarbon pigments, the carotin of green plants. In addition it has been shown that the milk fat carotin nearly always has associated with it one or more minor constituents whose general properties and characteristics are identical with the xanthophyll group of pigments. Two and possibly three xanthophyll constituents were found in one sample of high colored butter fat.

In addition to the establishment of a chemical relation between carotin and xanthophylls and the yellow lipochrome of milk fat, it has been possible to demonstrate a much more significant fact, namely that this lipochrome whose origin has hitherto been considered to be in the animal body is in reality merely the carotin and xanthophylls of the food, which are absorbed by the body and subsequently secreted in the milk fat. Numerous feeding experiments show that when the food is deficient in carotin and xanthophylls for a period of time, the milk fat slowly decreases in color and eventually approaches a colorless condition. The experiments also show that when foods rich in carotin and xanthophylls are given to a cow whose milk fat is deficient in lipochrome, the color of the milk fat at once increases in proportion to the amount of pigments fed. This is true regardless of whether the carotins and xanthophylls are associated with chlorophyll as in green feeds, or whether chlorophyll is completely absent and xanthophylls almost so, as in carrots.

The experiments show in addition that small amounts of carotin, such as are present in the oil of cottonseed meal have apparently no effect on the color of the butter fat. It is not clear, however, whether this is due to the smallness of the amount of carotin or to the state in which it exists in the food, i. e., dissolved in oil. There is some evidence on both sides. Mendel and Daniels[1] have recently found

1. Jour. Biol. Chem. 13, No. 1, p. 72 (1912).

that when fifteen grams per day of Sudan III dissolved in oil was fed to a cow for three successive days, there was no indication of the dye in the milk. On the other hand, in the feeding experiments with Cow No. 301 where the bleached alfalfa hay was changed to timothy hay containing a small amount of carotin, there was also apparently no effect on the color of the milk fat.

It is especially noteworthy that all of the above feeding experiments which involved yellow corn are united in pointing to its inability to impart any color to the butter fat. This result is not so surprising, however, when viewed in the light of the character of the pigment of yellow corn as shown in the chemical studies. It was found there that the pigment is largely a xanthophyll. It may be stated that the butter fat of Cow No. 301 during the last yellow corn experiment failed to show the presence of the corn xanthophyll, when subjected to careful examination.

The feeding of carotin in the form of carrots to a cow giving as low colored milk fat as Cow No. 301 gave an excellent opportunity to study the proportion of carotin and xanthophyll in the resulting well-colored butter fat. This investigation was reported in connection with the study of the proportion of carotin and xanthophyll in butter fat under varying conditions of production. It was found that the xanthophylls were practically absent from the fat. The conclusion is that the xanthophylls must be present in the food in large excess, as in grass, before they will appear in the butter fat.

It was mentioned above, and the fact is worthy of special notice, that when the carotin and xanthophylls are withdrawn from the food the falling off in color of the butter fat is sometimes very slow. In the case of Jersey cow No. 57, it required twenty-seven days for the butter fat to drop in color from 43 to 8.5 units of yellow. Normally, it should require much less time for the color to drop this amount. For instance it required only 12 days for a similar drop to be brought about in the color of the milk fat of Jersey cow No. 59. In explanation of this difference it may be stated that upon a normal plane of nutrition, the blood serum furnishes the pigment for the milk fat. When the plane of nutrition is below normal in a lactating cow the body fat is drawn upon to aid in the production of milk fat and also for other purposes. If the body fat thus utilized has a high yellow color, as is usually the case in Jersey and Guernsey cows, the normal storage of pigment for the milk fat will be continually, at least partially, replenished. The reduction in color of the milk fat will then be much slower than normal. It was stated above and will bear repetition, that we have here an explanation of why Jersey cows apparently often produce high colored milk fat on a low pigmented ration, as during the winter months.

The results of these experiments are of considerable practical importance. It is readily seen, for instance, that the peculiar popular conception of the enhanced value of butter on account of a high yellow color is absolutely without foundation. It is furthermore seen that the prevailing opinion among some cattle breeders that Guernsey and Jersey cows are able to synthetically produce a high colored butter fat under all conditions is also unfounded. It has been shown that all breeds of cows will produce well-colored butter fat under proper feeding conditions. The reverse has been shown to be especially true, namely that a cow, regardless of breed, cannot produce high colored butter fat under normal conditions, unless the food contains the pigments which are utilized for that purpose.

With our present knowledge, however, we would not be justified in saying that there is no breed characteristic in connection with the color of butter fat. Under apparently equal conditions Jersey and Guernsey cows usually give higher colored milk fat than Holstein or Ayrshire cows. We have been able to offer some evidence, however, showing that during a moderate pigmentation of the milk fat, this difference largely disappears when the amount of fat produced and the proportion of the ration which is the source of the pigment are taken into consideration. Further experiments would be required to ascertain whether this is true under all conditions of milk fat pigmentation or only true for moderate pigmentation. The data already at hand at least entirely justify the statement that the so-called breed characteristic has been given more emphasis than is warranted by an actual study of the facts.

The results of our experiments are furthermore of considerable physiological significance. A direct source for the lipochromes of the cow has been established, which opens the question of a similar source for all animal lipochromes. The lipochrome of milk fat has been increased or decreased with great ease by merely varying the food of the cow. Such a result throws great doubt upon any physiological significance which the lipochromes have been supposed to exert in the animals in which they have been found. Apparently it is merely a question of the inability of the animal body to throw off the excess of carotin and xanthophylls contained in its food. Experiments will reported in a later paper of this series showing that the blood serum, of the cow at least, very rapidly takes up the carotin (especially) of the food and carries it through the body in combination with an albumin of the serum.

SUMMARY.

1. The fat of cows' milk owes its natural yellow color to the pigments carotin and xanthophylls, principally carotin, the well-known, wide-spread, yellow vegetable pigments found accompanying chlorophyll in all green plants.

2. The carotin and xanthophylls of milk fat are not synthesized in the cow's body, but are merely taken up from the food and subsequently secreted in the milk fat.

3. When food practically free from carotin and xanthophylls, such as the cow usually receives during the winter months, is given to a milk-giving cow, the immediate supply of these pigments in the organism is greatly depleted and may be entirely used up, on account of the constant drain upon the supply by the milk glands. The butter fat accordingly approaches a colorless condition in proportion to the supply of carotin and xanthophylls in the system, the length of time these pigments are kept out of the food, and also, very probably, in proportion to the amount of milk fat being produced.

4. If food rich in carotin and xanthophylls is given to a milk-giving cow whose milk fat has become practically colorless by reason of the above conditions, the organism will at once recover its lost pigments and the milk fat will increase in color in proportion to the amount of carotin and xanthophylls, especially carotin, in the food. Fresh green grass probably being the richest in carotin of all natural dairy cattle feeds, accordingly produces the highest colored butter.

5. There is some difference among different breeds of dairy cows in respect to the maximum color of the milk fat under equally favorable conditions for the production of a high color. Each breed of cows, however, will undergo the same variation in color of the milk fat which follows a withdrawal or addition of carotin and xanthophylls, especially carotin, to the food. Under some conditions, also, the apparent breed characteristic largely disappears. The popular opinion in regard to the breed characteristic has been overemphasized, and statements in regard to it should in the future be qualified with a statement of the conditions of feed, etc.

6. Under normal conditions cows of all breeds produce very high colored milk fat for a short time after parturition. The pigments of the fat at this time are identical with the normal pigments of the fat. Their increase at this time is probably due to the physiological conditions surrounding the secretion of the milk of the freshening animal.

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CAROTIN—THE PRINCIPAL NATURAL YELLOW PIGMENT OF MILK FAT.*—PART III.

The Pigments of the Body Fat, Corpus Luteum and Skin Secretions of the Cow.

LEROY S. PALMER AND C. H. ECKLES

Recent investigations in regard to yellow animal pigments have shown that some of them are closely related chemically or identical with yellow pigments of plant origin. Willstätter and Escher [1] have found that the pigment of egg yolk is isomeric with a crystalline xanthophyll of green plants, and Escher [2] has found the pigment of the corpus luteum to be identical with the widely distributed hydrocarbon pigment, the carotin of fruits, flowers and green plants.

In a study of the commonly observed yellow lipochrome of butter fat we have found [3] that it is composed principally of a pigment identical with carotin, with one or more minor constituents which are evidently identical with the xanthophyll pigments. We have furthermore shown by an unbroken chain of evidence that these pigments are present in the milk fat as a result of feeding a ration containing an abundant amount of these pigments. The presence of these pigments in milk fat is therefore not due to any synthetic powers which the animal possesses, but merely to the fact that the organism absorbs the pigments along with the products of food digestion and subsequently secretes them dissolved in the milk fat. We were accordingly able to vary the amount of pigment in the milk fat by simply choosing the proper feeds, i. e., either deficient in carotin and xanthophylls or very rich in these pigments. The above relations between the carotin and xanthophylls of milk fat and the carotin and xanthophylls of feeds were found to hold good for all breeds of dairy cows.

1. Zeit. f. Physiol. Chem. 76, pp. 214-225 (1912).
2. Zeit. f. Physiol. Chem. 83, p. 198 (1913.
3. Research Bulletin No. 10 Missouri Agr. Exp. Sta; Jour. Biol. Chem. 17, p. 191 (1914).

*See Foreword, Part I, for statement of co-operation with Dairy Division, U. S. Department of Agriculture.

The establishment of the chemical identity of the pigment of milk fat and of its simple physiological relation to the carotin and xanthophylls of green plants at once opened the question of a similar relation of the pigment which is so often observed in the body fat of cows, especially those of certain breeds, such as the Jersey and Guernsey. The question is also raised as to the presence of xanthophylls in the corpus luteum pigment. In addition an interesting question is opened as to whether the yellow skin secretions of certain breeds of dairy cows, which is often interpreted as indicating the ability of these animals to secrete yellow milk fat, is also due to the same pigments that characterize the butter fat.

The present investigation was undertaken for the purpose of studying these questions. In addition some information was gathered relative to the relation of the breed of the cow to the amount of color found in the body fat.

METHODS OF IDENTIFICATION.

The general methods of studying and identifying the pigments of the body fat, corpus luteum and skin secretions of the cow were the same as were used in the study of the pigment of milk fat. A detailed account of these methods may be found in the preceding paper of this series, which deals with the milk fat pigment.

These methods were a study of what we have called the spectroscopic, solubility, and adsorption properties of the pigments. The methods were confined to characteristic, physical and chemical properties of the pigments for the same reasons that they were used for the study of the milk fat pigment, namely because, in the case of the body fat at least, the very large amount of fat with which the pigments are associated precludes their isolation in sufficient quantity for chemical analysis. In the case of the pigments of the corpus luteum and skin secretions not enough material was available for isolating any great quantity of pigment.[1]

The Spectroscopic Properties.—It was found that carotin and xanthophylls isolated from green alfalfa hay, carrots or other plants rich in these pigments showed characteristic absorption bands when viewed in a spectroscope of narrow dispersion. When the spectroscope was set at an arbitrarily chosen standard, each class of pigments exhibited bands in characteristic position, especially in carbon bisulphide solution, and could then be readily identified. The arbitrary

1. Escher succeeded in isolating less than 0.5 gram of impure crystals of corpus luteum pigment from 10,000 cows' ovaries.

standard was obtained by fixing the arbitrary scale attached to the spectroscope at a constant figure which was furnished by a sodium flame, the spectrometer slit being closed to furnish the narrowest possible line. This standard did not of course give absolute measurements of absorption bands but merely a means of comparing the position of bands of various solutions, which was the desired end in view. Before measuring the bands of an unknown pigment, the strength of its solution was adjusted to give bands of as nearly the same intensity and clearness as the bands whose arbitrary measurements furnished the standard. The arbitrary standards for the absorption bands of carotin and xanthophylls, which were adopted, are given in Table I.

TABLE 1.—SPECTROSCOPIC STANDARDS OF CAROTIN AND XANTHOPHYLLS.

Pigment	Solvent	Measurement of absorption bands.		
		Band I	Band II	Band III
Carotin	CS_2	225—242	261—278	301—319
Carotin	$C_2H_5(OH)$	257—275	303—318	345—364
Xanthophyll	CS_2	233—253	272—291	312—330
Xanthophyll	$C_2H_5(OH)$	263—280	305—325	355—...

The Solubility Properties.—The relative solubility properties of carotin and xanthophylls are based on the fact that organic compounds are best soluble in solvents of similar composition. Accordingly carotin, which is a hydrocarbon, is much more soluble in hydrocarbons like petroleum ether than in the alcohols. Similarly the xanthophylls are much more soluble in the alcohols than in a hydrocarbon like petroleum ether. These phenomena, as stated in the preceding paper of this series, were discovered and elaborated by Tswett [1] and by Willstätter and Mieg.[2] At this station they were found to be very characteristic of carotin and xanthophylls and in addition very useful for separating and differentiating the carotin and xanthophyll constituents, not only of plants but also of the milk fat pigment. Thus the xanthophyll constituents of a mixed pigment could be readily separated by shaking the petroleum ether solution of the

1. Ber. d. Deut. Botan. Gessel. 24, pp. 316, 384 (1906); 29, p. 630 (1911).

2. Ann. d. Chemie. 355, p. 1 (1907).

mixed pigment with eighty to ninety per cent alcohol. Or if an eighty to ninety per cent alcoholic solution of the mixed pigments was shaken with petroleum ether, the latter solvent would completely extract the carotin, leaving the xanthophylls in the alcohol. By the second method especially, it was possible to show the presence of xanthophyll pigments in butter fat which could not be extracted from their alcoholic solution by petroleum ether.

The Adsorption Properties.—These properties were discovered by Tswett.[1] They are based on the fact that carotin and the xanthophylls show a great difference in regard to the ease with which they enter into combination with certain finely divided organic and inorganic compounds, such as Inulin, Saccharose or $CaCO_3$ For instance, carotin is not adsorbed at all by $CaCO_3$ from its perfectly anhydrous carbon bisulphide or petroleum ether solutions, while the xanthophylls are adsorbed to a greater or less extent. Briefly then, it has been found that if a carbon bisulphide solution (in which the pigments have an unusually brilliant color) of the mixed pigments is filtered slowly through a column of $CaCO_3$, previously moistened with the solvent, the pigments will be differentiated into zone-like rings as they pass through the column, depending on their adsorption affinity towards the $CaCO_3$. Carotin being unadsorbed will pass through first as a rose or red orange colored zone, with the various xanthophylls distributed above as zones of different shades of yellow or orange. The xanthophylls which are completely adsorbed by the $CaCO_3$ can be washed out afterwards by a stream of petroleum ether containing ten per cent absolute alcohol.

THE PIGMENTS OF THE BODY FAT.

The pigment of the body fat of the cow has never been subjected to a critical examination. Newbigin[2] reports the only attempt to identify it. He extracted the pigment from a sample of bright yellow body fat and compared its properties with those of a yellow pigment which he isolated from the salmon, which pigment, he says, belongs to a widely distributed group of animal pigments commonly confounded with the lipochrome pigments. He found the body fat pigment very similar in properties to the yellow non-lipochrome pigment. It did not give the lipochrome properties and was very little soluble in methyl alcohol. Newbigin also compared the body

1. Ber. d. Deut. Botan. Gessel. 24, pp. 316, 384 (1906).

2. D. Noel Patton, Report of Inv. on Life History of Salmon (1898), Article XN, p. 159.

fat pigment with the pigment from maize, with the result that, "The maize pigment gave the lipochrome reaction faintly with H_2SO_4, distinctly with HNO_3 while the fat pigment gave no lipochrome reaction. In other respects, in tint, solubility, etc., the pigments closely resembled each other." The experience of this station in studying the lipochrome properties of the milk fat pigment seems to indicate that Newbigin's results were due to the fact that he was working with decomposed pigment. Nevertheless particular attention was paid to the lipochrome properties of the body fat pigment.

Method of Isolation.

The method was the same as that used for isolating the milk fat pigment. It consisted in careful saponification of the fat with alcoholic potash (2 c.c. of 20% solution per gram of fat) and subsequent extraction of the unsaponifiable matter from the diluted soap (3 volumes of water to one of soap) with ether. The ether extract was sometimes purified by re-saponification and re-extraction with ether, and sometimes was freed from cholesterol with digitonin. Only small amounts of fat were used for each test, i. e., 25 to 30 grams, as the studies of the milk fat pigment showed that the best results could thus be obtained.

Identification of Pigments.

Only two typical experiments showing the character of the body fat pigments will be reported.

Experiment I.

Twenty-five to thirty grams of pure rendered kidney fat from a Jersey cow was used for this experiment. The fat had a high yellow color testing in the one-inch tintometer cell 54 yellow, 2.3 red. The unsaponifiable matter of the fat had a golden yellow color in ether solution and a blood red color in carbon bisulphide solution. The carbon bisulphide solution left no adsorbed zone in the $CaCO_3$ when analyzed chromotographically, but passed through unadsorbed as an orange-red or rose colored zone. A very small amount of pigment was left in the column, however, which was readily washed out by a stream of alcoholic petroleum ether.

The main pigment was now examined with respect to its solubility relations toward petroleum ether and 80 per cent alcohol. A very minor constituent was thus obtained more soluble in 80 per

cent alcohol than in petroleum ether (b. p. 30-50). This constituent, when combined with the pigment adsorbed by the $CaCO_3$ in the chromotogramm, did not show sufficiently sharp absorption bands for accurate measurements.

The main petroleum ether soluble pigment was transferred to carbon bisulphide in which it showed two strong bands and a third faint one, the measurements of which are given in Table 2. The residue from this solution gave a greenish-blue color with concentrated sulphuric acid.

Experiment II.

An unusually high colored fat taken from the back of a Jersey cow was used for this experiment. The pure rendered fat tested in the one-inch tintometer cell 80 yellow, 2.7 red. Thirty grams of this fat was saponified with 300 c. c. of 5 per cent alcoholic potash by dissolving the fat in the hot alkaline solution, letting stand for 24 hours in the cold with frequent shaking and finally boiling on the steam bath for one half hour. By using this procedure not a trace of foreign pigment was developed. One extraction with ether rendered the diluted soap colorless. The golden yellow ether extract, after purification, was concentrated into 50 c. c. of absolute alcohol. On careful analysis of this solution it was found possible to separate its pigment into a major pigment which was readily extracted by petroleum ether and a minor pigment which could not be extracted by petroleum ether. The main petroleum ether soluble pigment was readily soluble in carbon bisulphide with a blood red color, and in this solvent showed two strong absorption bands and a third faint one, the measurements of which are given in Table 2. Analyzed chromotographically, the carbon bisulphide solution passed through as an unadsorbed beautiful rose colored zone. There was no differentiation. The residue from the solution gave a deep blue color with concentrated sulphuric acid.

The alcohol soluble pigment, which probably comprised several per cent of the total, was transferred to ether by diluting the alcoholic solution with much water in a separatory funnel. Petroleum ether was added, precipitating some water, and the ethereal solution washed with water until clear. The solution was now evaporated and the yellow residue dissolved in carbon bisulphide, giving a yellow-orange solution which showed two fine absorption bands, and a third fainter one, the measurements of which are given in Table 2. The bands seem to be shifted more toward the blue than the usual xanthophyll bands.

The orange-yellow carbon bisulphide solution was now analyzed chromotographically. Only one pigment was present, which passed through the column very slowly as a narrow orange zone, leaving no pigment in the $CaCO_3$ which could be washed out with alcoholic petroleum ether.

TABLE 2.—ABSORPTION BANDS OF CAROTIN AND XANTHOPHYLLS OF BODY FAT.

Experiment No.	Measurements of absorption bands			
	Carotin		Xanthophylls	
1.	Band I	223—242	.....	
	Band II	259—279	.....	
	Band III	300—319	.....	
2.	Band I	224—243	Band I	235—252
	Band II	262—288	Band II	278—302
	Band III	302—322	Band III	315—335

It must necessarily be concluded from these experiments that Newbigin's "inert" class of lipochromes does not exist in the body fat of the cow, but, on the other hand, the pigment of this fat is, like the butter fat pigment, composed of a major carotin and one or more minor xanthophyll constituents, all of which also show the properties of lipochromes. It is to be noted also that the number of xanthophylls in the body fat varies, as was found to be the case in the butter fat studies.

The Relation Between the Color of the Body Fat and the Food of the Cow.

Numerous feeding experiments in connection with the study of the pigment of milk, reported in the preceding paper of this series, showed that the carotin and xanthophylls which were found to characterize the milk fat were present there on account of the fact that the food contained these pigments. Since the pigment of the body fat is also composed of carotin and xanthophylls it is natural to suppose that it is, like the pigment of milk fat, derived from the food, the carotin and xanthophylls being carried to the fat depots and fat synthesizing body cells in the same manner that they are carried to the milk glands.

In order to obtain evidence of this fact, however, the following experiment was undertaken. Two barren and dry Jersey cows in

moderate flesh were fed wheat straw alone for sixty days or until the animals had lost as much fat as was considered necessary for the second part of the experiment. The daily ration of cow No. 25 was then changed to 9 pounds of yellow corn and 20 pounds of green alfalfa hay, which was rich in carotin and xanthophylls. Cow No. 21 was given a daily ration averaging 11.4 pounds of white corn and 14 pounds of bleached clover hay, very deficient in carotin and xanthophylls. Cow No. 25 was slaughtered at the end of 81 days. Her gain in weight during this period was 160 pounds. Cow No. 21 was slaughtered at the end of 95 days. She had gained materially in condition during her "fattening" period although the scales showed little gain in weight. This was probably due to a much greater "fill" when receiving wheat straw. Samples of fat from various parts of the body were taken from each cow at slaughtering and used for color readings. The results are given in Table 3. The colorimetric readings in this and subsequent tables were made on the rendered, melted fat, measured by the Lovibond Tintometer. A complete description of this instrument may be found in the preceding paper of this series.

TABLE 3.—THE RELATION OF FEED TO COLOR OF BODY FAT.

Part of body.	Color of fat.					
	Cow No. 25.			Cow No. 21.		
	Yellow	Red	Light	Yellow	Red	Light
Rib plate	50	2.3	1.0	1.4	0.1	0
Caul	47	2.1	1.0	3.6	0.5	0
Thoracic cavity	29	1.3	1.0	8.0	1.0	0
Around ovaries and uterus	49	2.3	1.0	2.5	0.3	0
Attached to fourth stomach	33	1.6	1.0	24.0	1.7	1.0
In pelvic cavity	50	2.3	1.0	47.0	2.1	1.0
Kidney	54	1.6	1.0	50.0	2.1	1.0
Crops	50	2.3	1.0	47.0	1.9	1.0
Over last rib	47	2.3	1.0	50.0	2.1	1.0
Over outside chuck	47	2.0	1.0	47.0	1.8	1.0

The results of this experiment are even more striking when the amount of fat on the various parts of the bodies of the two cows is taken into consideration. Aside from the kidney fat and pelvic cavity fat, which were probably not disturbed to any extent during the starvation period, and which furthermore were of equal color in the two animals, the largest proportion of the entire fat

of the two cows was represented by the caul fat. The caul apron of Cow 25 had a net weight of 12,132 grams; that of Cow 21 a net weight of 7,364 grams. It is here that the great difference in color is noticeable. In fact, if all the fat on the body of Cow 21 had been rendered and its color compared with the same from Cow 25, the difference in color would have been very marked.

There can be no doubt that the above data is conclusive as to the effect of feeding a non-pigmented ration to a fattening cow whose fat had been largely eliminated by starvation previous to the feeding of the colorless ration. In other words, it is apparent that the body fat of the Jersey cow is colored primarily because the food is rich in carotin and xanthophylls during the time the fat is on. The blood serum of both cows contained carotin at the time of slaughtering. Unfortunately no comparison was made of the amount in the serum of the two cows. No doubt there was a much smaller amount in the blood serum of Cow 21, than in the serum of Cow 25. In both cows there was no known path of elimination of the blood pigment during the starvation period, the cows being both dry and barren. During the fattening period of Cow 25, the demands made on the blood store by the body fat producing cells, was replenished by the food. In the case of Cow 21, however, there was no replenishing source, and the amount in the serum must have greatly diminished.

The data resulting from this experiment have some significance aside from the relation of the body fat to the food of the cow. The fact that the outside fats of the two cows were of equal color, and the inside fats, especially the caul and rib plate fat, were of such widely different colors, would seem to indicate what fats are drawn upon first in starvation in this class of animals, and what fats are laid on first during fattening.

Relation Between Color of Body Fat and Breed of Cow.

Considerable attention was given in connection with the study of the milk fat pigment, to a study of the relation of the breed to the amount of pigment in the fat. This study showed that the relation of breed to milk fat coloration is a relative one, Holstein and Ayrshire cows producing well-colored butter fat as well as Jerseys and Guernseys under proper feeding conditions. It was also shown that under certain conditions there was no difference in milk fat coloration among the different breeds. These results naturally raised the question whether a similar study of the body fat pigmentation

would lead to the same conclusions. It is not uncommon to find the body fat of Jersey and Guernsey cows with a high yellow color. This has led to a general belief that this phenomenon is a characteristic of only these breeds of cows. As a matter of fact butchers and also the consumer look with disfavor upon beef from these animals on account of this high color of the fat. Although Table 3, above, shows very clearly that the color of the body fat of Jersey cows is as much dependent upon the feed as the color of the milk fat, it was nevertheless important to study the coloration of the body fat, of the different breeds, which had accumulated under ordinary conditions. In this way the normal breed relation could be determined.

Only a few animals were available for this study. Besides the data for the two Jersey cows given in Table 2, we have the colorimetric study of the body fat of one Jersey and 3 Holstein cows. The data from these animals is given in Table 4.

TABLE 4.—RELATION OF BREED TO COLOR OF BODY FAT.

Part of body	Cow No. 2, Jersey		Cow No. 207, Holstein		Cow No. 226, Holstein		Cow No. 221 Holstein	
	Yellow	Red	Yellow	Red	Yellow	Red	Yellow	Red
Rib plate	54	1.5	47	1.7	..	..	15.0	1.2
Crops	63	1.8	14	1.2	..	..	21.0	1.2
Thoracic cavity	17	1.0	36	1.5	9	1.0	6.0	1.2
Caul	50	1.7	54	1.7	18	1.1	12.0	1.2
Pelvic cavity	47	1.5	61	1.8	..	..	10.0	1.2
Over last rib	63	1.8	17	1.3	..	..	23.0	1.2
Ovaries, uterus	..	..	62	1.8	..	..	..	..
Chuck	54	1.7	14	1.0	..	..	22.	1.2
Kidney	47	1.5	64	1.8	..	..	20.	1.2
Stomach	..	..	..	..	24	1.0	11.	1.2

The most important points presented in this table are the wide difference between the color of the fats of Holstein Cow No. 207 and the other two Holstein cows; and the wide difference between the color of the inside and outside fats of Holstein Cow No. 207. The first point is possibly due to an individual characteristic of Cow No. 207, although it is not known under what conditions the fat was formed. It should be stated in connection with the data of Cow No. 226 that the animal died in parturition, and the reason so few samples of fat are recorded is due to the fact that the animal had no fat on the body at those particular places. In regard to the

data on Cow No. 207, it is to be noticed that the inside fat all had a color equal or greater than the corresponding fat of Jersey Cow No. 2, while the outside fats were uniformly much lighter in color. In explanation of this result it may be said that milk cows are known to lay on fat first on the inside of their body, and we have data to show, not only that this particular Holstein cow normally produced high-colored milk fat under favorable feeding conditions, but also that the laying on of most of the fat, whose color is shown in the table, was during the summer when her ration was largely fresh green grass.

This does not hold true for Holstein Cow No. 221, for much of her fat was also laid on while on grass. It should be added too that the milk fat of this cow was never known to have a very high color. This was brought out especially in a carrot-feeding experiment with this animal which was reported in the preceding paper of this series. The maximum color obtained in that experiment was practically the same as the maximum color found in her body fat. There seems to be a breed characteristic evident here, but owing to the high color readings obtained from Holstein Cow No. 207, it may be due to the individual rather than to the breed.

Perhaps the most important point brought out by this data is that the color of the body fat of any individual, regardless of breed, laid on under given feeding conditions is practically the same as the color of the milk fat under the same conditions.

Another point which should be mentioned here, but which will be more readily understood in the light of the results which will be given in a subsequent paper, is that this difference between individuals is not due to lack of carotin in the blood. The amount of carotin in the blood of Cow No. 221 at the time of slaughtering was as great as is found in the blood of a Jersey cow receiving the same feed.

Our data is not sufficiently extensive to warrant any conclusions as to the normal difference in body fat pigmentation between the different breeds. Very probably it is considerably greater than the normal difference between the milk fat pigmentation of the different breeds. The reason for this is not evident from our present knowledge of the physiology of pigmentation. The fact that such a wide difference often does exist is of considerable importance, however, in explaining why the milk fat of Jersey and Guernsey cows often has a higher color than can be explained by the character of the ration. Reference was made to this in connection with the feeding experiments reported in the preceding paper on the milk fat pigment.

It was there shown that changing the ration of a Jersey cow from one rich in carotin and xanthophylls to an unpalatable one very poor in these pigments did not result in an immediate lowering of the color of the butter fat, but resulted rather in a gradual reduction in color, extending over a considerable period of time. The animal at the same time usually lost weight. This fact taken in connection with the normal high color of the body fat of Jersey cows which was brought out in the present experiments, gives a clear explanation of the entire phenomenon. The pigments of the body fat were being drawn upon, or rather the utilization of the body fat for energy liberated pigments which furnished a partial temporary supply for the milk fat. The milk fat of cows, whose body fat lacked this high color would, therefore, under similar conditions lose color very much faster. The high color of the milk fat of Jersey cows on a nonpigmented ration is, therefore, due to the fact that their body fat has a normal high yellow color.

THE PIGMENTS OF THE CORPUS LUTEUM.

Viewed in the light of the foregoing investigations it is not surprising that Escher [1] has found that the corpus luteum pigment belongs to the carotin group, thus establishing its identity with the principal milk fat and body fat pigments. In view of the plurality that has been established for both the milk and body fat pigments it became at once important to study the corpus luteum pigment in this connection also. Only a few corpora lutea were available for the study, in fact the ovaries of only six cows at different times were available for examination and in three cases only were well-developed corpora lutea found. Of Jersey Cows No. 21 and No. 25 slaughtered at the same time, only Cow No. 25 had a corpus luteum of any development. Jersey Cow No. 8 and a Hereford cow were slaughtered at the same time but only the beef bred cow had a well-developed corpus luteum. Cow No. 207 slaughtered at another time had no well-developed corpus luteum but there were the remains of a number of former corpora lutea and one just developing. Holstein Cow No. 221 slaughtered some time later, had a well-developed corpus luteum.

The investigations of the corpora lutea of the Jersey cows, Nos. 21 and 25, were carried out on the combined pigments previous to the discovery of the xanthophyll constituent of butter fat pigment, but the data obtained is nevertheless very instructive.

1. Loc. cit.

The corpora lutea were carefully cut away from the surrounding tissue, ground up with sand, and extracted with ether. In this solvent and in alcohol, the pigment showed two absorption bands in the blue part of the spectrum. Solubility tests on the alcohol solution showed that petroleum ether and carbon bisulphide extracted *almost* all the pigment. That which was not extracted was treated with hot alcoholic potash and the soap extracted with ether in which the pigment all readily went. The saponified pigment was transferred to alcohol and freed from cholesterol with digitonin. After concentrating, the cholesterol-free filtrate was extracted with carbon bisulphide. Not all the pigment was extracted even with two extractions, and petroleum ether extracted no color from the remaining light-yellow alcoholic solution. The experiments with the secondary pigment were not carried farther at this time as the significance of its presence was not appreciated, but viewing the data in the light of the results of the milk fat and body fat investigations it is evident that a secondary xanthophyll pigment was present here. This has been emphasized because the results of the investigations subsequently conducted were unfortunately vitiated because of unexpected aldehyde resin colorations which developed during saponification. It was shown by a special study that these reddish yellow bodies when present in considerable quantity are extracted from the diluted alkaline solutions by ether, but are not readily extracted from alcohol by petroleum ether. Consequently they interfere with a proper study of the pure pigments. Such a result was obtained in the study of the corpora lutea pigments of Cow No. 8 and the Hereford cow. The only noteworthy result of that investigation was to obtain a beautiful rose colored unadsorbed zone in a chromotogramm of a carbon bisulphide solution of the combined pigment. This solution showed three absorption bands, the measurements of which are given in Table 5.

The next investigation was with the corpora lutea of Holstein Cow No. 207. As stated above there was no well-developed corpus luteum, the largest part of the pigment obtained being from the remains of several former corpora lutea which were present as small red colored patches about the size of a pin head. These were carefully cut out and macerated with a little sand and $CaSO_4$ and extracted with carbon bisulphide for several hours. The solution, of about 25-50 c. c. volume, had a deep orange-red color, which showed three beautiful bands, the third band being considerably fainter than the first two. The measurements are given in Table 5.

A chromotogramm of this solution showed only one rose colored zone which passed rapidly through the $CaCO_3$ column. A little of the solution was evaporated into absolute alcohol and after making the alcohol eighty per cent, the pigment was studied in regard to its solubility properties toward petroleum ether (b. p. 30-50° C) and carbon bisulphide respectively. In both cases the alcohol was left absolutely colorless. In this case then, where the pigment was chiefly from the remains of former corpora lutea, carotin was the only pigment present.

TABLE 5.—ABSORPTION BANDS OF CORPUS LUTEUM CAROTIN IN CARBON BISULPHIDE.

Bands	From Hereford Cow No. 8. Good corpus luteum.	From Holstein Cow No. 207. Remains of corpus luteum.,
Band I	225—242	225—242
Band II	262—282	262—285
Band III	305—320	305—320

The final investigation was with the well-developed corpus luteum from Holstein Cow No. 221. Both ovaries of the cow were ground up with sand and plaster of paris and the mass extracted with petroleum ether (b. p. 30-50° C.) until the extract was colorless. The pigment thus extracted was carefully differentiated between the petroleum ether and 85 per cent alcohol. No pigment whatever was extracted by the alcohol. The pigment was then submitted to saponification with KOH after transferring to alcohol. No aldehyde resin pigments formed during saponification. The pigment was extracted from the soap with ether. The ether extract was thoroughly washed with water as usual and then concentrated at a low temperature with the constant addition of petroleum ether so that the pigment finally remained in petroleum ether solution. This solution was then shaken with 85 per cent alcohol. The alcohol extracted no pigment whatever. In this case then, although a normal corpus luteum was used, and the entire pigmented extract submitted to saponification, carotin was the only pigment present.

The result of this study was to confirm the results of Escher[1] that the corpus luteum pigment is identical in properties with carotin. In addition we have shown that this pigment, like the principal pigments of milk fat and body fat, may have associated with

1. Loc. cit.

it small quantities of xanthophyll pigment. It is possible, however, that these xanthophylls are present in the fat which may be extracted along with the carotin of the corpus luteum.

THE PIGMENTS OF THE WAXY SECRETIONS IN THE EARS AND ON THE SKIN OF JERSEY COWS.

It was stated in the introduction that the secretions of the skin of Jersey and Guernsey cows is often considered as indicating the ability of these breeds to secrete yellow milk fat. It was accordingly thought that a brief investigation of this pigment would be of interest and possibly of some scientific value.

The yellow skin secretion of Jersey cows is especially abundant in the ears. A few grams of the yellow waxy matter was accordingly scraped from the ears of several pure bred Jersey cows and the wax macerated with ether, which readily dissolved away the pigment and some fatty matter, giving a bright yellow solution. The ether solution was concentrated to low volume and diluted with about 100 c. c. of 2 per cent alcoholic potash and the solution boiled on the steam bath for 30 minutes. The pigment was extracted from the soap solution with ether in the usual way. The extraction of the pigment was easy and complete. The ether solution was freed from alkali as usual and then diluted with some petroleum ether. The slightly cloudy solution which resulted was washed with water until clear and evaporated into absolute alcohol. The alcohol was now diluted with petroleum ether (b. p. 30 to 50° C) and water added sufficient to cause separation. Several extractions with petroleum ether resulted in the division of the original pigment into a major petroleum ether soluble pigment and a minor pigment which could not be extracted from 80-90 per cent alcohol with petroleum ether.

The petroleum ether pigment gave a red orange carbon bisulphide solution showing the carotin absorption bands:

I. 224—243
II. 263—287
III. 303—320

and a beautiful rose colored unadsorbed zone in the $CaCO_3$ chromotogramm.

The 80 per cent alcohol soluble pigment which amounted to two or three per cent of the entire pigment, gave a yellow-orange carbon bisulphide solution. The solution showed only one absorption band however, the other bands being obscure.

I. 232—254

It is thus seen that the yellow pigment of the skin secretions of the Jersey cow is identical with the other yellow lipochromes of the body and like them belongs chiefly to the carotin group of pigments.

THE BODY FAT AND BLOOD SERUM PIGMENTS OF THE NEW-BORN CALF.

Carotin and xanthophylls having been found to be normal constituents of the body fat of cows which had been fed green feeds or other feeds containing an abundant amount of these pigments, an interesting question was raised as to whether these pigments are present in the body of the new-born calf. If these pigments should be found to be entirely absent from the new-born calf, additional proof would therefore be offered that these pigments were the result of subsequent feeding. The presence of carotin and xanthophylls in the new-born calf, however, would not be proof that these pigments cannot arise from the food, but would merely indicate that they were able to traverse the placental barrier from the mother whose body is normally rich in these pigments. In this connection the question would be especially interesting in view of the fact that Mendel and Daniels [1] have recently found that fat soluble dyes, such as Sudan III, do not traverse the placental barrier of small animals such as cats and rats, whose milk fat and body fat, however, is readily tinted as the result of feeding the dyes.

In order to study this question, the following experiment was carried out:

A new-born pure bred Jersey calf weighing 50 pounds was not allowed to suckle its mother but was slaughtered a few hours after its birth.

Five hundred c. c. of the blood was caught in a cylinder and allowed to clot. After standing 48 hours, 250 c. c. of serum was obtained. The proteins were precipitated from the serum with alcohol and were filtered off on a Bücher funnel with suction. They had a reddish gray color. They were rubbed up to a paste with absolute alcohol in a mortar and then extracted with boiling absolute alcohol. The extract was absolutely colorless. The alcoholic filtrate from the precipitated proteins had a greenish-yellow color. It was concentrated to 50 c. c. and absolute alcohol added, precipitating a little protein. The filtrate had a beautiful greenish-yellow color but the pigment was not extracted by carbon bisulphide, petroleum

1. Jour. Biol. Chem. 13 No. 1, p. 72 (1912).

ether, or ether, but seemed to be partly thrown down by lead acetate and by saturation of a dilute alcoholic solution with $(NH_4)_2SO_4$. Acid mercuric nitrate solution decolorized the alcoholic solution at the same time throwing down a white precipitate.

The only conclusion that can be drawn from this experiment is that the blood serum of the new-born calf is free from carotin. A small amount of an unknown pigment was present which was readily soluble only in alcohol, and insoluble in water, ether, carbon bisulphide, and petroleum ether.

There was practically no fat on the body except a little around the kidneys and in the tissue of the caul apron. In the body the latter tissue had a slight brownish color. All the fat tissue that could be obtained was ground up, rendered and filtered. About 40 grams in all was obtained. The rendered fat had a slight yellow color giving a tintometer reading in 1-inch layer of 4 yellow and .3 red. When solid the fat had a greenish-yellow tint. Thirty grams of the fat was saponified with alcoholic potash and the soap extracted with ether. It was possible to differentiate the small amount of pigment thus obtained so that it was about equally divided between petroleum ether and 80 per cent alcohol. In carbon bisulphide solution these portions showed their relation to carotin and xanthophyll both in the spectroscope and chromotogramm. Both portions showed two beautiful bands which measured as follows:

Body Fat Carotin (In CS_2)		Body Fat Xanthophyll (In CS_2)	
Band I	225—244	Band I	235—250
Band II	263—280	Band II	270—285

The results of this experiment show that a small amount of carotin and xanthophyll are present in the body fat of the new-born Jersey calf. The results present the apparent anomaly, however, of the presence of the pigments in the body fat and their absence in the blood. In explanation of this it may be said that the body fat of the new-born calf, the amount of which is very small indeed, probably arises from the fat of the mother, being transferred to the foetus a very small quantity at a time. The small quantity which would be present in the blood stream under these conditions, i. e., dissolved in fat, would not have been detectable by the method used for the investigation of the pigments of the blood serum. It is absolutely certain that there were none of these pigments present in the blood serum in the way in which they are normally found in the mature animal.

The results of the investigation are of further value in indicating that under proper feeding conditions, it might be possible to raise even a Jersey cow with practically none of the characteristic carotin and xanthophyll pigments in her body.

DISCUSSION OF RESULTS.

The results of the experiments reported in this paper are in perfect accord with those of the preceding paper. The discovery of the carotin and xanthophyll nature of the milk fat pigment would lead quite naturally to the supposition that the other lipochrome pigments of the body of the cow are of the same character. This supposition was fully borne out by the result of experimental study. The yellow lipochromes of the body fat, of the corpus luteum and of the skin secretions were found to be composed principally of carotin with one or more minor xanthophyll constituents.

In addition to the establishment of the chemical relation of these pigments to the carotin and xanthophyl of green plants in the case of the body fat at least it has been possible to show that the pigments are derived from the food in a manner identical with pigments of the milk fat. The carotin and xanthophylls of the corpus luteum and skin secretions must therefore be derived from the same source.

Viewing the results from a physiological standpoint, it is seen that the establishment of such a source for these pigments and the ease with which they are therefore increased and decreased,[1] throws great doubt upon any physiological significance which these pigments have been supposed to exert in the animal body. In the case of the corpus luteum for instance, the accumulation of the carotin during the formation of this body is merely a phenomenon incidental to the rupture of the Graafian follicle and the subsequent formation of the cellular tissue around the central blood clot, and to the fact that the blood serum is normally very rich in carotin, as will be shown in the following paper. This, of course, does not explain the mechanism of the accumulation of the cărŏtiñ-containing cells around the ruptured Graafian follicle. The chemical combination of the carotin in the blood serum is no doubt of importance in this connection.

The popular opinion that the body fat of Jersey cows is normally characterized by a higher yellow color than Holstein cows has been at least partially confirmed by experimental study, although it

1. This is especially true of the milk fat and, as will be shown in the succeeding paper, the blood serum.

was found that Holstein cows may also possess high-colored body fat. At least there seems to be more breed characteristic in this respect, than in the case of the pigmentation of the milk fat. There is no foundation, however, for the belief that beef has a lower value because its fat has a high color. If this pigment is the same as is demanded by the consumer for butter, why should not beef with high-colored fat also be more desirable? It is recognized of course that some of the unfavorable attitude toward beef with highly colored fat arises partially from the fact that it indicates that the beef probably came from a dairy cow. The two ideas are nevertheless very closely associated.

The normally high color of the body fat of Jersey cows and also of those of the Guernsey breed, explains why cows of these breeds often appear to be producing well-colored butter on a ration deficient in carotin and xanthophylls. Several statements in regard to this have already been made. This will bear repetition, however, because the subject is an important one. Briefly, it may be said that when cows whose body fat has a high yellow color are put upon a ration deficient in carotin and xanthophylls and also, as is usually the case with such rations, deficient in food value, the body fat is called upon to furnish energy value for the animal and also in many cases to supplement the food digestion products in the production of milk fat. It is readily seen that in such cases an important source is opened up for pigments for the milk fat. Just how important this source could be would depend upon the amount of highly colored body fat available for the needs of the body, and upon the rapidity with which it would be used up. If our experimental data are correct showing that the inside fats, such as the caul fat and rib plate fat, are the first drawn upon in starvation of this class of animals, then the amount of available highly colored fat would be rather large. Dairy cows usually have a fairly abundant amount of these fats, especially the caul fat. It is thus readily seen that a continuous drawing upon these inside fats for a long period of time would result in a very slow and gradual reduction of the color of the milk fat. The deduction that the animal was actually producing colored milk fat on a carotin-xanthophyll-free ration would, therefore, be quite natural but nevertheless entirely false.

In a similar manner it is readily seen why the breeders of Jersey and Guernsey cattle have been led to believe that the yellow skin secretions of these breeds are indicative of their ability to produce yellow milk fat. It is interesting to find that the yellow pigments of these secretions are carotin and xanthophylls. It should be clearly

borne in mind, however, that the only indication that a cow will secrete yellow milk fat is that the food contains an abundance of carotin and xanthophylls.

SUMMARY.

1. The yellow lipochrome of the body fat, corpus luteum and skin secretions of the cow, like the lipochrome of butter fat, is composed principally of a pigment whose physical and chemical properties are identical with the carotin of green plants. The same pigment may have associated with it one or more minor constituents whose physical and chemical properties are identical with the xanthophylls of green plants.

2. The carotin and xanthophyll pigments of the body fat are derived from the food of the cow. The body fat of Jersey cows formed on a ration deficient in carotin and xanthophylls, is devoid of color.

3. The body fat of Jersey and Guernsey cows is usually characterized by a higher yellow color than cows of other breeds. This is of great importance in explaining why cows of these breeds may sometimes show a much slower elimination of the pigment from milk fat on a non-pigmented ration, as during the winter months. In these cases the body fat furnishes a supplementary source of pigments for the milk fat.

4. The yellow body fat of Jersey and Guernsey cows should not be a point against the use of these animals for beef. The pigments here are the same as those for which the consumer will pay a higher price when present in butter.

5. The breeders of Jersey and Guernsey cattle are probably correct in their belief that the yellow skin and skin secretions of these animals are characteristic of the breeds. It is not correct, however, that this characteristic is indicative of the ability of these animals to secrete yellow milk fat under all conditions. The only indication of this is whether the food contains an abundance of carotin and xanthophylls.

6. The blood serum of the new-born Jersey calf is free from carotin and xanthophylls. The small amount of fat on the body is tinted very faintly with these pigments.

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CAROTIN—THE PRINCIPAL NATURAL YELLOW PIGMENT OF MILK FAT—Part IV. *

A. The Yellow Pigment of Blood Serum.
B. Carotin and Xanthophylls During Digestion.
C. The Pigments of Human Milk Fat.

LEROY S. PALMER and C. H. ECKLES.

A. THE YELLOW PIGMENT OF BLOOD SERUM

Very few investigations have dealt with the so-called lutein of the blood serum. Thudichum [1] was the first to mention it and classify it as a lutein. Schunck,[2] a number of years later, showed that the lutein of fowl serum was spectroscopically identical with the L. xanthophyll which he isolated from yellow flowers and green plants. Halliburton [3] also studied the lutein of the serum of the hen, but the pigment isolated by him had evidently lost its spectroscopic properties in view of Schunck's investigation. Finally, Krukenberg [4] extracted the lutein from ox serum by shaking with amyl alcohol. The extract showed two absorption bands. He used the designation lipochrome for the pigment. His work is usually mentioned in the present text books of physiological chemistry.

Recent investigations in connection with various animal luteins or lipochromes have shown that they may be classified as belonging to the widely distributed carotin or xanthophyll groups of pigments of the vegetable world. Willstätter and Escher [5] have identified the pigment of egg yolk as an isomer of the crystalline xanthophyll of green plants; and Escher [6] has shown that the principal corpus luteum pigment is identical with the carotin of plants. Extending this work

*See Research Bulletin No. 9, p. 312, for statement of Co-operation with U. S. Dept. of Agriculture.

1. Proc. Roy. Soc. 17, p. 253 (1869).
2. Proc. Roy. Soc. 72, p. 165 (1903).
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5. Zeit. f. Physiol. Chem. 76, pp. 214-225 (1912).
6. Zeit f. Physiol. Chem. 83, p. 198 (1913).

we have shown that the yellow lipochromes of the milk fat and body fat of cows are also composed principally of carotin, altho both have associated with them one or more minor xanthophyll constituents. In addition we have shown conclusively that these pigments originate from the food of the cow. They are therefore not products of animal synthesis but merely substances assimilated with the digestion products of the food and subsequently secreted in the milk fat or laid up in the body fat. The studies leading to these results are given in the two preceding papers of this series.[1]

Up to this time the experimental evidence pointing to the above stated physiological relation between the carotin and xanthophylls of plants and the pigments of butter fat and body fat has been based upon feeding experiments in which the relation between the amount of these pigments in the food and the color of the milk fat and body fat was carefully studied. It was recognized however that the evidence would not be absolutely complete until the means of transporting the food pigments to the milk fat and body fat could be established. A close study of the yellow lipochrome of the blood serum, in a manner similar to the preceding studies of the milk fat and body fat pigments, naturally seemed to offer the most ready means of establishing the physiological relation between the plant pigments and the lipochromes of milk fat and body fat.

The present investigation was therefore undertaken for the purpose of studying the yellow lipochrome of the blood serum in regard to its chemical and physiological relations to the carotin and xanthophylls of green plants and to these pigments when found in the milk fat and body fat of the cow. It was believed that this investigation would serve the twofold purpose of establishing the connecting physiological link between these plant and animal pigments and also scientifically classifying the blood serum lutein of the cow which pigment has never been the subject of close investigation.

METHODS OF IDENTIFICATION.

The methods used for identifying the pigment of the blood serum were the same as were used in the study of the milk fat and body fat pigments. They consisted in the application to the isolated pigment of the characteristic physical and chemical properties of carotin and xanthophylls. These properties were the position of the spectroscopic absorption bands, the relative solubility toward petroleum ether and 80 to 90 per cent alcohol, and the adsorption affinity toward

1. Also Jour. Biol. Chem. 17, No. 2, pp. 191, 211 (1914).

calcium carbonate. A detailed description of these properties when applied to both the plant carotin and xanthophylls and the pigments of milk fat, body fat, corpus luteum and skin secretions of the cow was given in the two preceding bulletins [1] of this series, and need not be repeated here. The measurements of the spectroscopic absorption bands of the carotin and xanthophylls which are used for comparison were made according to an arbitrarily fixed and standard scale. It may not be out of place therefore, to repeat here a table which was given in the paper immediately preceding this one, showing these standard measurements. This table is given below as Table 1. The measurements in carbon bisulphide solution only are given.

TABLE NO. 1.—SPECTROSCOPIC STANDARDS OF CAROTIN AND XANTHOPHYLLS.

Pigment	Solvent	Measurements of absorption bands		
		Band I	Band II	Band III
Carotin	CS_2	225—242	261—278	301—319
Xanthophyll	CS_2	233—253	272—291	312—330

METHODS OF ISOLATION.

The study of the blood serum lutein required considerable preliminary study of methods of isolation. The amyl alcohol method of Krukenberg [2] was not considered suitable on account of the high boiling point of the solvent. The method used by Schunck [3] seemed to be much better suited for the work. He precipitated the proteins from the serum with alcohol, and as the proteins carried down the lutein he was able to isolate it by extracting the precipitated proteins with boiling absolute alcohol. Preliminary investigations of the blood serum lutein using Schunck's method showed, however, that it was applicable only to serum free from dissolved red blood corpuscles. When hemoglobin was present it was always carried down with the protein and some of the red color dissolved in the subsequent alcohol extract. In addition, the method did not seem to be a quantitative one, some of the lutein invariably being found in the dilute alcoholic filtrate from the precipitated proteins. These investigations showed however, that in every case both petroleum ether and carbon bisulphide almost quanti-

1. Research Bulletins Nos. 10 and 11, Missouri Agr. Exp. Sta.; also Jour. Biol. Chem. 17, pp. 191, 211 (1914).
2. Loc. cit.
3. Loc. cit.

tatively extracted the yellow pigment from its alcoholic solution on dilution with a little water. This result at once indicated the carotin nature of the blood serum lutein, and this was confirmed by the experiments reported below.

The methods used for isolating the pigment in these studies varied somewhat in detail but were all based upon a preliminary, more or less complete dessication of the blood serum by calcium sulphate (plaster of Paris). The details are given in connection with the report of the experiments.

CHEMICAL IDENTIFICATION OF THE PIGMENT.

The following experiments were conducted to show the chemical relation of the blood serum lutein to the carotin and xanthophylls.

Experiment I.

About 20 cubic centimeters of golden yellow serum from Jersey Cow No. 8* was mixed with plaster of Paris until almost dry, dried for a few minutes on the steam bath, the mass pulverized, and shaken with successive volumes of petroleum either in an Erlenmeyer flask until no more color appeared in the petroleum ether. The extract was light yellow in color and no color was extracted from the concentrated solution by 80 per cent alcohol. The plaster of Paris mass was now shaken with successive proportions of petroleum ether containing 10 per cent absolute alcohol, until the extraction was colorless. The resulting extract had a deep yellow color containing many times as much pigment as the extract with petroleum ether alone. This solution, after concentration, was extracted with 80 per cent alcohol. Apparently no color was extracted. The petroleum ether solutions were combined, evaporated to dryness and the residue dissolved at once in carbon bisulphide giving a deep red-orange solution which showed 3 absorption bands, Band III being much fainter than the other two. The measurements of the bands are given in Table No. 2.

Experiment II.

Fifty cubic centimeters of the same serum was completely dessicated with plaster of Paris and the pulverized mass shaken with absolute alcohol and ether until no more color was extracted. The ether was distilled off and the golden-yellow alcoholic solution saponified with

*Note:—The serum in this case and in all subsequent cases was obtained by allowing the freshly drawn blood to clot in a tall cylinder or jar and the serum which pressed out on standing syphoned off into glass stoppered bottles.

KOH. After dilution with water, the soap was extracted with ether. The ether was washed, filtered and evaporated into absolute alcohol. The alcohol was diluted to an 80 per cent solution and extracted with petroleum ether (b.p.30-50°C.) until no more color was extracted. The alcohol layer was left with quite a little color, but by far the greatest part of the color was in the petroleum ether extracts. The petroleum ether soluble pigment gave a deep red carbon bisulphide solution which showed 3 absorption bands, the third being faint. The measurements of the band are given in Table No. 2.

The 80 per cent alcohol soluble pigment showed no clear absorption bands.

Experiment III.

Fifty cubic centimeters of the same serum was mixed to a thick paste with plaster of Paris, and the pasty mass shaken thoroughly with 700 c.c. of hot 95 per cent alcohol. All the color was extracted, a second extraction with fresh alcohol being colorless. The yellow extract was concentrated to 100 c.c. An equal volume of 10 per cent alcoholic potash was added and the solution boiled on the steam bath for one hour. No aldehyde resin pigments formed. The alkaline solution was diluted with 3 volumes of distilled water and extracted with two-thirds of its volume of ether. All the color was extracted by the one extraction. After washing and filtering, the golden-yellow extract was evaporated to dryness and the residue taken up at once with petroleum ether (b.p.30-50°C.) This solution was now thoroughly shaken with 80 per cent alcohol until no more color was extracted. Fresh petroleum ether extracted a little color from the alcoholic extract, which was not re-extracted by fresh 80 per cent alcohol. The blood serum lutein was now divided into a major and minor pigment, the major being insoluble in 80 per cent alcohol in the presence of petroleum ether and the minor being insoluble in petroleum ether in the presence of 80 per cent alcohol.

The petroleum ether soluble pigment had a blood-red color in CS_2 solution and showed 3 absorption bands, the measurements of which are given in Table No. 2.

Analyzed chromotographically it passed through $CaCO_3$ unadsorbed as a beautiful rose-colored area, leaving no adsorbed zones.

The 80 per cent alcohol soluble pigment was transferred to ether by diluting its alcoholic-ether solution with much water, and from the ether to carbon bisulphide after evaporation of the former. In carbon bisulphide it gave an orange-yellow solution showing two absorption bands in the 25 m.m. cell. The measurements of the bands are given in Table No. 2.

Chromotographic analysis showed the presence of only one pigment which very slowly passed through the $CaCO_3$ as a yellow zone.

Experiment IV.

Serum from Jersey Cow No. 2, to the amount of 275 cubic centimeters, was dessicated with a little more than the calculated amount of plaster of Paris necessary to take up the water, and after setting over night the hard mass was pulverized in a mortar. The powder was moistened with 95 per cent alcohol and shaken with petroleum ether (b.p.30-50°C.) until the petroleum ether extracted no more color, and then with ether until that extract was colorless.[1] The petroleum ether extract was concentrated to 50 c.c. and the solution added to the alcohol-free ether-alcohol extract, which had been concentrated to about 150 c.c. An equal volume of 4 per cent alcoholic potash solution was now added to the combined ethereal solutions, the ethers evaporated off and the alcoholic solution boiled on the steam bath for a few minutes. The pigment was then extracted from the alkaline alcoholic solution in the usual way and when in alcoholic solution was analyzed with respect to petroleum ether and eighty to ninety per cent alcohol. Two pigments were thus obtained with proportions of perhaps 95 and 5 per cent of the total.

The petroleum ether soluble pigment gave a red colored residue which dissolved instantly in carbon bisulphide with a blood-red color, and showed the most beautiful absorption bands yet seen for this pigment. Three bands were visible, the third band being considerably fainter than the other two bands but clear and distinct. The measurements are given in Table No. 2.

1. The mechanism of this method of obtaining the blood serum pigment is so interesting, its advantages so striking and its results so satisfactory, that it is worthy of some discussion.

It appears that the addition of just sufficient alcohol (either absolute or 95 per cent) to thoroughly moisten the dessicated serum liberates the main lutein pigment in such a way that when the moistened mass is shaken with petroleum ether the result is the same as if an 80 per cent to 90 per cent alcoholic solution of the isolated pigment is shaken with petroleum ether. There is the additional advantage however, that the $CaSO_4$ prevents the formation of emulsions and holds the alcoholic solution so firmly fixed in the paste that the petroleum ether can be poured away and the use of a separatory funnel be entirely dispensed with. When all the pigment more soluble in petroleum ether than in 80 per cent alcohol is thus extracted, any pigment which remains can be readily extracted with ether which mixes readily with the dilute alcohol. The pigment thus extracted can be readily freed from alcohol by shaking with water leaving the last as well as the first pigment extracted, in low boiling point solvents, an additional decided advantage in view of the ease with which they are oxidized. It should be added however that the method for extracting the pigment more soluble in alcohol than in petroleum ether does not apply well for serum containing much haemoglobin for in this as well as all other alcohol methods the red pigment is somewhat soluble in the dilute alcohol.

Analyzed by means of a chromotogramm the solution showed only a wide, quickly filtering, unadsorbed, rose-colored zone.

The 80 per cent alcohol soluble pigment was transferred to ether and then to carbon bisulphide, giving in the latter an orange-yellow solution showing one band and much end-absorption. In the chromotogramm it showed 2 zones close together, an upper orange zone and a lower canary yellow zone. The carbon bisulphide solution of the orange zone showed 2 absorption bands and end-absorption. The measurements are given in Table No. 2.

TABLE No. 2.—SPECTROSCOPIC ABSORPTION BANDS OF BLOOD SERUM CAROTIN AND XANTHOPHYLLS.

Experiment No.	Solvent	Absorption bands	
		Carotin	Xanthophylls
1	CS_2	I. 225—242 II. 263—286 III. 305—322	
2	CS_2	I. 223—243 II. 262—285 III. 302—325	
3	CS_2	I. 225—242 II. 263—286 III. 305—325	I. 232—254 II. 273—295 III.
4	CS_2	I. 223—242 II. 262—286 III. 300—320	I. 232—252 II. 270—292 III. 310—

DISCUSSION OF EXPERIMENTS.

It must be concluded from the above experiments that the principal lipochrome of the blood serum of the cow is identical with that of the milk fat, body fat, and corpus luteum, and as in the case of these pigments, with the carotin of green plants.

It appears also from the above investigations that a small portion of the blood lutein pigment is composed of xanthophylls. It was found to be much more difficult to show their presence in the blood serum than in the body fat or butter fat. The reason for this is not perfectly clear but a close study of the investigations throws some light on the question. It will be noticed that it required complete extraction of

a comparatively large amount of serum with ether and subsequent saponification of the fat thus extracted to really demonstrate the presence of xanthophylls. It is a well-known physiological fact that the proportion of fat in blood serum is comparatively small.* When coupled with the above observations, this seems to indicate a relation between the xanthophylls and the fat carried by the blood. Some observations which will be reported later, in connection with the fate of the carotin and xanthophylls during digestion, will furnish more evidence in this same direction.

PHYSIOLOGICAL RELATION BETWEEN CAROTIN OF BLOOD SERUM AND FOOD OF COW.

After establishing the chemical relation between the principal blood serum lipochrome and the carotin of the food, it became important to establish a similar relation from a physiological standpoint. Very fortunately this was recognized previous to conducting some of the important feeding experiments which showed the relation between the color of milk fat and the food of the cow, and which were reported in Research Bulletin No. 10,[1] Missouri Agr. Exp. Sta., the second bulletin in this series. It was accordingly arranged to study the variation in the amount of carotin and xanthophylls in the blood serum during portions of these feeding experiments. In this way it could be determined what relation exists between the amount of carotin and xanthophylls in the blood serum and the amount of these pigments in the milk fat, as well as the relation between the amount of carotin and xanthophylls in the serum and amount of these pigments in the food. Such a study required the devising of some method of analysis whereby the color of the various blood serums could be compared with each other and also with the color of the butter fat. The following method was adopted.

In the case of live cows whose blood was to be tested, a trocar was inserted in the jugular vein and 200 to 250 c. cm. of blood drawn off into a glass cylinder. As soon as the blood had clotted and sufficient serum had pressed out, two 10 cc. portions were pipetted off and carefully dessicated with an excess of plaster of Paris. The powdered mass was moistened with absolute alcohol, and the color extracted immediately by shaking with the selected solvent until colorless. For one sample the solvent was ether and for the other sample the solvent was petroleum ether. In all the studies the petroleum

*Note:—Hammerstein gives .1 to .7%.

1. Also Jour. Biol. Chem. 17, p. 191 (1914).

ether extract proved to be the easiest to handle as it was practically free from water. The extract in each case was carefully evaporated to a volume of 1 to 2 cubic centimeters and then made up to 12½ cubic centimeters with absolute alcohol, this volume being just sufficient to fill the one-inch cell of the Lovibond Tintometer. The solutions were analyzed at once in the Tintometer and their color readings recorded. Duplicate determinations were thus obtained. This was considered necessary since the method was not free from error due to possible bleaching of the extracted pigments. The entire procedure was carried out as quickly as possible. The results of the duplicate determinations were averaged.

The first series of observations of the color of the milk fat and blood serum corresponding to various pigmented rations was made with Ayrshire cow No. 301. These feeding experiments and the resulting variation in the color of the milk fat were shown in detail in Tables 12, 13 and 14 of the preceding bulletin dealing with the milk fat pigment. The relation between the character of the ration, and the color of the milk fat and blood serum at stated intervals during the feeding experiments is shown in Table No. 3, below.

TABLE No. 3.—THE RELATION OF THE CHARACTER OF THE RATION TO THE COLOR OF THE MILK FAT AND BLOOD SERUM.
AYRSHIRE COW No. 301.

Date of sample.	Feed of cow	Butterfat		Serum	
		Yellow	Red	Yellow	Red
1913					
Jan. 7	Cottonseed meal and cottonseed hulls only	1.3	0.4	3.3	0.5
Jan. 24	Cottonseed hulls, timothy hay and white corn	1.2	0.4	2.6	1.1
Feb. 7	Timothy hay, cottonseed hulls, cottonseed meal and yellow corn	2.0	0.5	4.9	1.2
Mar. 1	Timothy hay, cottonseed hulls, cottonseed meal, yellow corn, and 20 lbs. carrots per day	24.0	1.3	54.0	1.8
Mar. 6	Timothy hay, cottonseed hulls, cottonseed meal, yellow corn, and 20 lbs. carrots per day	24.0	1.4	47.0	1.5
Mar. 27	Timothy hay, cottonseed hulls, cottonseed meal, and yellow corn	7.0	1.0	26.0	0.7

The above table shows in a very striking manner that the amount of carotin in the blood serum of the lactating cow, as well as the amount of carotin and xanthophylls in the milk fat, is dependent upon the ration. The figures in the table also show very clearly why the butter fat in the first two cases was not absolutely colorless. These two samples were taken at the end of a long-continued feeding of a ration almost entirely lacking in carotin and xanthophylls, which was planned for the purpose of eliminating as far as possible the color from the milk fat. It is evident that this was not accomplished in the strictest sense of the word because the blood serum in both cases still contained a small amount of carotin.

The results of these studies were so striking that it was considered advisable to confirm them if possible with other animals. We fortunately had at hand, 6 cows of 3 different breeds, all pure bred animals, which were in ideal condition for such an experiment. They had all just completed an experiment in which their feed for 12 to 14 weeks had been essentially a non-pigmented one, being made up of cottonseed meal, corn stover and very light-colored timothy hay. A night and morning milking of each cow was combined and after determining the percentage of fat in the combined sample [1] the milk was separated, the cream churned, and the color of the rendered butter fat observed in the Tintometer. The same day, samples of blood were drawn from each animal and the color of the serum determined by the method described above. The feed of the cows was now changed so that it was largely made up of alfalfa hay, rich in carotin and xanthophylls, and later a little fresh green pasture grass. Thirty days after the cows had been on this feed the first experiment was repeated and the color of the butter fat and blood serum again observed. The results of this experiment are given in Table No. 4.

1. The weight of the combined sample was also recorded.

TABLE No. 4.—RELATION BETWEEN CHARACTER OF RATION AND AMOUNT OF PIGMENT IN MILK FAT AND BLOOD SERUM.

Cow No.	Breed	Date	Grams fat.	Butterfat		Serum	
				Yellow	Red	Yellow	Red
213	Holstein	3–11–13	122	8.5	1.4	6.0	0.7
213	Holstein	4–10–13	135	54.0	1.8	48.0	1.1
220	Holstein	3–11–13	167	3.0	0.7	7.0	0.8
220	Holstein	4–10–13	208	22.0	1.2	41.0	1.0
303	Ayrshire	3–11–13	213	2.5	0.6	11.0	0.9
303	Ayrshire	4–10–13	263	16.0	1.1	40.0	1.0
16	Jersey	3–11–13	304	11.0	1.7	10.0	0.9
16	Jersey	4–10–13	363	64.0	2.0	45.0	1.1
57	Jersey	3–11–13	240	5.2	1.2	13.0	1.1
57	Jersey	4–10–13	263	54.0	1.7	57.0	1.8
64	Jersey	3–11–13	281	4.7	1.5	7.5	0.7
64	Jersey	4–10–13	350	47.0	1.6	45.0	1.0

The results of this experiment are as striking and conclusive as in the experiment with Cow No. 301, and show that there is a direct relation between the amount of carotin in the food and the amount of lutein in the blood serum, just as there is a direct relation between the presence of an excess of carotin in the food and the production of a high-colored butter fat. It is necessarily true also that there is a direct relation between the color of the butter fat and the amount of lutein in the blood serum. A small amount of lutein in the blood serum will always mean a light-colored butter fat. It does not appear to be necessarily true, however, that a high-colored serum will be accompanied by a high-colored butter fat. The only conclusion in this connection that can be drawn from the data of Tables 3 and 4 is that an increase in the color of the blood is accompanied by an increase in the color of the fat. The actual color of the fat under these conditions is apparently dependent upon a number of conditions which are not explained by this data. The amount of fat being produced and the breed of the animal are both factors which probably influence the color of the fat. There is certainly a wider difference

between the color readings of the butter fats than between the color readings of the blood serum extracts during the second part of the experiment whose data are given in Table No. 4.

It is possible that this difference may be explained on the ground that the albumin content of the milk is in some way closely related to the color of the milk fat. At any rate the data given in the following table admit of such interpretation. The conditions of the animal preceding the data were as follows: Cow No. 301 had been subjected to severe underfeeding, i. e., she received only about 70 per cent of the food required to produce her milk and maintain her body weight. The food she received during this time was composed of about 5 pounds of a mixture of corn, bran and linseed meal, and about 7 pounds of alfalfa hay, a ration moderately rich in carotin. Her ration was then changed to one practically free from carotin, consisting of white corn, cottonseed meal and bleached alfalfa hay. The cow was brought back to a normal plane of nutrition on this ration. The immediate effect on the composition of the milk and the color of the milk fat is shown in Table 5. The subsequent effect upon the color of the milk fat is given in Table No. 15 of the preceding Bulletin of this series which dealt with the milk fat pigment.

TABLE No. 5.—A POSSIBLE RELATION BETWEEN THE ALBUMIN OF MILK AND THE COLOR OF THE MILK FAT.
AYRSHIRE COW No. 301.

Date 1912	Hay Lbs.	Grain Lbs.	Milk fat per day Grams	Total protein Grams	Casein Grams	Albumin Grams (c)	Color of fat	
							Yellow	Red
9–23–24	7.2(a)	4.9	254	209	138	37	15	1.8
9–25–26	8.1(b)	4.9	249	203	134	36	15	1.8
9–27–28	11.0	6.0	262	222	138	47	19	1.8
9–29–30	14.0	7.0	272	230	135	57	21	1.7
10–1–2	14.5	7.5	267	237	132	67	28	1.8
10–3–4	9.8	8.0	276	220	139	46	20	1.7

(a) Alfalfa hay, rich in carotin.
(b) Bleached alfalfa hay, free from carotin.
(c) Calculated.

The data in Table No. 5 shows that although the conditions of feed were such that a decline in color would be expected, the reverse was found. The point to be emphasized is that the sharp increase in the color of the milk fat was coincident with an increase in the

albumin content of the milk. The result points to the probability of a relation between the higher color and the increase in albumin. Additional evidence pointing to the same relation is the presence of extremely high color along with a very abnormal amount of albumin in colostrum milk, as pointed out later in this paper.

An attempt was made to determine if any definite relation exists between the albumin and color as found in the milk of various individuals. The milk of 12 cows representing the Jersey, Holstein and Ayrshire breeds was used. The feed received was pasture grass and some grain. The color of the milk fat and the percentage of albumin were determined for each animal. The results of the study are given in Table No. 6.

TABLE NO. 6.—RELATION BETWEEN THE ALBUMIN CONTENT OF MILK AND THE COLOR OF MILK FAT, UNDER NORMAL CONDITIONS.

Breed	Cow No.	Pounds milk per day	Grams protein per day	Grams albumin per day	Grams fat per day	Color of fat.	
						Yellow	Red
J.	14	18.4	311.0	24.45	459.0	64.0	2.7
J.	64	12.9	254.0	22.65	380.2	64.0	2.8
J.	57	10.8	202.0	22.02	267.0	64.0	3.0
A.	303	20.2	310.0	33.20	363.5	50.0	1.7
H.	221	15.1	270.0	27.05	257.0	43.0	1.8
H.	222	14.5	233.8	20.12	202.5	33.0	1.3
J.	16	20.8	339.0	35.75	414.0	64.0	3.1
J.	317	13.0	234.5	41.00	259.0	80.0	3.5
H.	225	13.3	200.0	22.35	150.9	47.0	1.8
H.	220	16.3	525.0	35.17	192.5	24.0	1.7
A.	301	18.7	282.5	39.00	273.0	54.0	2.0
H.	213	11.4	161.5	19.05	144.2	64.0	2.7

"J." stands for Jersey, "H." for Holstein, "A." for Ayrshire.

There appears to have been no relation between the albumin of the milk and the color of the milk fat of these animals. It is not considered however, that these results are conclusive either as proving or disproving the supposition that such a relation exists. Our knowledge of the subject is too limited at present to enable us to control all the factors that enter into the question.

THE TRANSPORTATION OF CAROTIN AND XANTHOPHYLLS BY THE BLOOD SERUM.

When it had been shown conclusively that the lutein of the blood serum of the cow is composed of the carotin and xanthophyll pigments of the food, taken up along the digestive tract and transmitted by means of the blood to the fat synthesizing cells of the milk glands

and body tissue, it became a matter of considerable importance to ascertain how the blood carries these pigments through the body. It has already been shown that there is a strong possibility that the minor constituent of the pigment, i.e., the xanthophylls, are carried in the serum dissolved in the fat. When considering such physiological phenomena as the great volume of blood in circulation in an animal as large as the cow and the rapidity with which it circulates, it would seem very probable that even the very small percentage of fat in the blood is sufficient to account for all the pigment, both carotin and xanthophylls, which is presented to the milk glands and body cells. On the other hand, when one considers the very large proportion of carotin which is present in any given quantity of blood serum of a cow receiving a ration rich in carotin, it must be concluded that the fat plays little if any part in the transportation of this pigment. The studies that are to be presented will, therefore, consider only the carotin of the blood serum, since it is this pigment that comprises by far the greatest proportion of the lutein of the serum.

It might be considered that the carotin is carried by the serum merely in simple solution. In fact, Thudichum [1] stated that the lutein of the blood is in solution in the serum. This seems to be very probable especially in view of the fact that Krukenberg [2] found that it could be extracted from the serum by means of amyl alcohol.

Many facts can be presented, however, that go to show that the carotin does not exist in the serum in simple solution. In the first place neither carotin from plants nor the carotin of the serum itself are, when isolated, taken up to any extent when treated with the pure blood serum. Blood serum almost free from carotin from natural causes, showed no indication of having taken up the carotin in either case when poured over the pure amorphus pigments; and the serum itself showed no increase in the amount of color that could be extracted by petroleum ether after dessication with plaster of Paris and moistening with alcohol.

In addition to the above the following observations were made: [3]

1. Five c.c. portions were shaken vigorously with equal volumes of petroleum ether, ether, CS_2 and amyl alcohol respectively. All extracts were colorless except in the case of the amyl alcohol which was golden-yellow, and showed the carotin absorption bands both

1. Loc. cit.
2. Loc. cit.
3. Except where stated most of work about to be reported was done with a golden-yellow, high-colored serum from Ayrshire cow No. 301. The serum was obtained from this cow by drawing 250 c.c. of blood from the jugular vein and allowing it to clot and the serum to press out. The serum was free from red corpuscles.

in carbon bisulphide and alcohol, the solution in the first solvent showing three distinct bands. This shows that the lipochrome which Krukenberg extracted from ox serum was the pigment which we have identified as carotin. On addition of alcohol to the other mixtures the solvents in each case completely extracted the pigment on shaking.

2. Five c.c. portions were dessicated with plaster of Paris and shaken with ether, petroleum ether, and carbon bisulphide, respectively. A mere trace of color was extracted in each case. When a little absolute alcohol was added however, in all cases the solvents became well colored.

3. Five c.c. of serum was diluted with twenty volumes of water, without causing any precipitation of the pigment.

4. Twenty-five c.c. of serum was treated with successive portions of saturated $(NH_4)_2\ SO_4$ solution to the following per cent saturations: 28-35, 36-40, 45-46, and finally to one-half saturation. The fractionally precipitated globulins were in every case practically free from carotin, and the half saturated globulin free serum was golden-yellow. The color was entirely precipitated from a portion of this solution on complete saturation with $(NH_4)_2\ SO_4$. The remainder was acidified with a few drops of 1½ per cent acetic acid and heated to about 80°C. The coagulated albumins carried down only a small part of the color. The entire pigment was precipitated from the filtrate, however, on complete saturation with $(NH_4)_2\ SO_4$ in substance, the light precipitate which came down being deep yellow in color. This deep yellow precipitate was readily soluble in water giving a clear yellow aqueous solution from which neither ether nor petroleum ether would extract any color until the protein in the solution had first been coagulated with alcohol.

5. Five c.c. of serum was diluted to 25 c.c. with distilled water and the solution saturated with $Mg\ SO_4$ in substance. The globulins were filtered off. The filtrate was golden-yellow. Acetic acid was added to a concentration of 1 per cent. The precipitated albumins were bright yellow, leaving the solution colorless. Petroleum ether and carbon bisulphide extracted a slight amount of color from this precipitate on long contact. After the addition of a little alcohol, however, both solvents readily extracted the color.

6. One hundred c.c. of serum from Jersey Cow No. 25 was diluted with several volumes of water, a pinch of NaCl and a few drops of glacial acetic acid added and the solution heated quickly to a temperature just below the boiling point. The coagulum which formed contained a very little pigment but the filtrate was golden-yellow. No color could be extracted from the filtrate by carbon bisulphide, or by

ether, even after making strongly alkaline with potassium hydroxide. Amyl alcohol extracted the pigment.

7. Experiment 6 repeated on 200 c.c. of the same serum gave the same result. The entire pigment in the golden-yellow filtrate was coagulated by boiling. The coagulum was not soluble in water.

8. Fifty c.c. of serum (Cow No. 2) was diluted to 350 c.c. with water, a pinch of salt added and the solution heated on the steam bath, with stirring until cloudiness appeared. On adding a few drops of glacial acetic acid, a sharp coagulation took place. On filtering, the filtrate was bright yellow in color. On saturation of the filtrate with $(NH_4)_2\ SO_4$ in substance, a comparatively small amount of deep yellow precipitate was thrown down leaving a colorless supernatant solution. The yellow precipitate, which was contaminated with a little $(NH_4)_2\ SO_4$, was readily soluble in water, giving a perfectly clear yellow solution from which the yellow color was again entirely thrown down on saturation with $(NH_4)_2\ SO_4$ in substance, or on the addition of mercuric nitrate. The latter precipitate when still moist would not give up its color to petroleum ether until first moistened with absolute alcohol. The bright yellow pigment now found in the petroleum ether gave a red-orange CS_2 solution which showed the three carotin absorption bands.

9. Two 350 c.c. portions of serum (Jersey Cow No. 2) were treated respectively as follows:

Portion A was treated with $(NH_4)_2\ SO_4$ in substance to one half saturation, according to the formula

$$X = \frac{VC_2}{18.158 - .54\ C_2}$$

Where V = original volume of protein solution.
C = desired saturation as grams in 10 c.c.
X = grams to be added to give the required saturation.

The globulins which precipitated carried down some of the pigment, but on dissolving them in 150 c.c. of warm water containing some $(NH_4)_2\ SO_4$, and adding $(NH_4)_2\ SO_4$ to half saturation, they were thrown down practically colorless. The yellow filtrate from this precipitation was added to the other globulin-free filtrate and the combined solutions diluted to 1500 c.c. with distilled water. This solution was now raised to a temperature of 75°C in a water bath. 15 c.c. of 1½ per cent acetic acid added and the temperature raised to 80°C, when a sharp coagulation occurred. The solution was filtered, giving a

perfectly clear bright yellow filtrate. It was saturated with $(NH_4)_2 SO_4$ in substance, throwing down a small amount of deep yellow precipitate. The precipitate was filtered off on a large (11-inch) Büchner funnel, using suction, so that the layer of yellow protein would be as free as possible from occluded $(NH_4)_2 SO_4$. The golden-yellow precipitate was sucked as dry as possible on the funnel and the sticky mass covering the paper in very thin layer dissolved in warm water, in which it was readily soluble, and the clear yellow solution set aside.

Portion B was diluted with an equal volume of distilled water, a little sodium chloride added, and the solution raised to a temperature of 75°C. in a water bath. Acetic acid was now added carefully until a heavy definite coagulation took place. The coagulated proteins carried down some of the pigment but by far the greatest part was in the clear yellow filtrate. This filtrate was saturated with $(NH_4)_2 SO_4$ in substance and the precipitated pigmented protein filtered off in the same way as in the case of portion A. After being made comparatively dry by suction, the deep yellow residue was readily soluble in a small amount of cold distilled water.

The two similar solutions from A and B were now combined and filtered on a small Büchner funnel through several layers of fine paper to free it from dirt and other foreign matter introduced by the $(NH_4)_2 SO_4$. The golden-brownish-yellow filtrate of about 250 c.c. volume had a faint cloudiness when viewed by transmitted light and contained some $(NH_4)_2 SO_4$. That the pigment of this solution was carotin was shown by the fact that when an equal volume of alcohol was added to 15 c.c. of the solution and the mixture was shaken with petroleum ether, the petroleum ether rose to the top as a golden-yellow solution, leaving the lower cloudy alcoholic layer colorless. The pigment in the petroleum ether layer gave a red-orange carbon bisulphide solution, and in this solvent showed the usual carotin absorption bands.

The aqueous solution was now dialysed in a parchment bag against running water for eight days. At the end of this time the solution was still giving a precipitate with barium chloride indicating that the solution was not free from $(NH_4)_2 SO_4$. No protein crystallization had occurred, but decomposition had begun, for the solution was cloudy, and showed a very fine coagulation. This coagulum was filtered off. It had a dirty brown color and when almost dry was quite sticky. It was not soluble in water, but both in the dry state and in suspension in water it gave up a golden-yellow color to ether, leaving the precipitate dirty white in color. The extracted pigment showed the three absorption bands of carotin in carbon bisulphide solution.

In the solid state the pigment was insoluble in absolute alcohol but readily soluble in petroleum ether—absolute alcohol, from which the petroleum ether readily extracted it on dilution with a little water. The solid pigment was also very difficultly soluble in alcoholic potash. After saponification for one half hour and extraction by ether, the pigment, in the solid state, was fused with a little solid sodium hydroxide and potassium nitrate in a porcelain crucible. The flux was dissolved in hot water and the solution evaporated to dryness on the steam bath in the presence of an excess of c. p. HNO_3. As much as possible of the residue was dissolved in hot water containing some c. p. HNO_3, the solution filtered and an equal volume of pure molybdate solution added to the 100 c.c. of filtrate. On digestion at 60°C, for several hours there was a distinct yellow precipitate of ammonium phosphomolybdate.

Returning to the slightly cloudy but yellow aqueous filtrate from the dialysed solution, we found that the color could be entirely thrown down; (1) by acid lead acetate as a light yellow precipitate which bleached almost entirely in 12 hours, but from which petroleum ether extracted a faint yellow color after soaking in alcohol for about one hour; (2) by nitric acid mercuric nitrate solution as a bright yellow precipitate which was very stable and gave up its color to petroleum ether only after soaking in alcohol;[1] (3) by neutral ten per cent solution of $AgNO_3$ as a deep yellow precipitate which was stable although darkening badly as the $AgNO_3$ oxidized in the light, but readily giving up its color to petroleum ether on addition of alcohol to the precipitate, the pigment thus extracted showing the carotin bands in CS_2 solution; (4) on saturation with $(NH_4)_2\ SO_4$ in substance as a deep yellow precipitate which was not soluble in water but when suspended in water gave up its color to petroleum ether only after the addition of absolute alcohol; (5) on addition of an excess of alcohol as a yellow precipitate which when dry gave up no color to petroleum ether alone, but to alcoholic petroleum ether gave up a yellow pigment which was quantitatively found in the petroleum ether on separation of the alcohol with a little water; (6) on heating the neutral solution to boiling as a yellow coagulum insoluble in water and giving up no color to hot alcohol or petroleum ether.

In addition to the above observations the following may be mentioned. In working with a large number of samples of blood serum

1. The pigment thus extracted showed the three carotin absorption bands in carbon bisulphide solution; in alcoholic solution it gave a pronounced precipitate of digitonin-cholesteride on addition of hot one per cent digitonin solution in ninety per cent alcohol.

it was often noticed that when the serum had stood for some time in a closed bottle in contact with a little supernatant air, an orange-yellow scum always came to the top. This was found to be a water insoluble protein which would not give up any color to petroleum ether when its aqueous suspension was shaken with that solvent, but when an equal volume of absolute alcohol was added and the shaking with petroleum ether repeated, the latter solvent rose to the top as a beautiful yellow layer. The pigment thus extracted gave a red-orange carbon bisulphide solution showing the three carotin absorption bands.

In order to show more conclusively the character of the protein with which the serum carotin is evidently combined, the coagulation temperature of the protein was determined. For this purpose 150 c.c. of serum was diluted with an equal volume of a saturated solution of ammonium sulphate. After filtering off the precipitated globulins, portions of the globulin-free filtrate were submitted to fractional coagulation. It was found that on carefully elevating the temperature to 80° C. and holding it at that temperature for a short time, the filtrate from the coagulated albumins still yielded a large amount of carotin on addition of alcohol and shaking with petroleum ether. On the other hand the coagulated albumins yielded a comparatively small amount of carotin. A similar result was obtained at temperatures of 81°, 82°, 83°, 84°, 85°, and 85.5° C., although the amount of carotin in the filtrate rapidly decreased with the increase in coagulation temperature. At 86° C., however, the pigmented protein had completely coagulated, and the filtrate yielded no carotin on treatment with alcohol and petroleum ether. The coagulation temperature limits of the pigment carrying protein therefore lie between 80° and 86° C., when the protein is in half saturated ammonium sulphate solution. There is no marked coagulation at the lower temperature, but it is completely coagulated at the upper temperature.

The coagulation temperature of the protein which carries the carotin in the blood was studied further with an aqueous solution of the protein obtained in a manner similar to the one used in obtaining the protein for the study previously reported. Briefly, an equal volume of saturated $(NH_4)_2\ SO_4$ solution was added to 200 cubic centimeters of blood, rich in carotin, from Holstein Cow No. 221. The globulins were filtered off and the golden-yellow filtrate heated carefully in a water bath to a temperature of 79° C. The coagulated proteins were filtered off. The yellow filtrate was saturated with $(NH_4)_2\ SO_4$ in substance and let stand several hours. The golden-yellow precipitate was filtered off on a Büchner funnel. After allowing to suck

quite dry, all crystals of $(NH_4)_2 SO_4$ were removed with a spatula and the protein dissolved in about 75 cubic centimeters of water, in which it was readily soluble. This deep yellow solution was neutral in reaction. It contained a small amount of $(NH_4)_2 SO_4$, the amount of which was determined quantitatively. $(NH_4)_2 SO_4$ was added to a portion of this solution to bring the concentration up to a normal solution. An equal volume of saturated $(NH_4)_2 SO_4$ solution was added to the remainder, giving a solution between 3 and 4 normal and one similar to the one whose coagulation temperature was observed above. The coagulation temperature of the pigmented protein of these two solutions was carefully studied. Both solutions were found to contain a small amount of colorless protein which coagulated between 65° and 75° C. This was filtered off, the filtrate retaining its original yellow color. This filtrate was then studied further.

The 3-4 normal or one half saturated $(NH_4)_2 SO_4$ solution acted in a manner identical with the solution whose study is recorded above. The first opalescence appeared between 79° and 80° C., and complete coagulation did not take place until the temperature was raised to 86° C.

It was not found possible to cause a clear coagulation of the pigmented protein in the neutral normal solution of $(NH_4)_2 SO_4$ even when the temperature was raised to 90° C. Opalescence began, however, between 82° and 82.5° C. Coagulation was readily obtained when the solution was heated to 89° C. in the presence of a very little HCl. (3 drops of a ½ normal HCl solution were added to 10 c. c. of solution.)

The large amount of evidence which has now been submitted in regard to the transportation of the carotin in the blood serum will justify but one conclusion, namely that the carotin exists in the blood in conjugation with one of the proteins. The evidence will also justify the conclusion that the protein with which the carotin is combined is an albumin.

Summarizing the evidence, we have shown that the carotin carrying protein is precipitated from its solution in the serum or from its aqueous solution, on complete saturation only with ammonium sulphate, or by saturation with magnesium sulphate only in one per cent acetic acid solution, or by heating its half saturated ammonium sulphate solution to 86° C.; the protein may also be coagulated by alcohol, or by boilding its solution in the presence of acetic acid. As in all salting out methods for the precipitation of proteins, the pigmented protein is readily soluble in water after being thrown down by ammonium

sulphate, but is no longer soluble in water after being coagulated by heat or alcohol.[1] The protein is not coagulated by saturation of its solution with sodium chloride.

Not very much can be said in regard to the character of the combination of the carotin with the albumin The combination is evidently a firm one, and is broken down only in the presence of alcohol so that the pigment can be extracted by ether or petroleum ether. The union is also broken down by dialysis, or at least rendered less firm, but is not broken down when the protein is precipitated as a lead, silver or mercury salt. It is interesting to notice that cholesterol and a phosphorus-containing body (probably lecithin) are mixed up in some way in the combination of albumin and carotin, the liberated carotin from the dialysed pigmented albumin yielding both cholesterol and phosphorus. We propose the name caroto-albumin or luteo-albumin for the chromo-protein which transmits the carotin from the food to the milk glands and fat synthesizing body cells of the cow.

The finding of this highly unsaturated hydrocarbon carotin pigment in combination with one of the albumins of the blood, probably similar to the combination of the haematin in the haemoglobin of the red blood corpuscles, at once raises some important questions as to a possible physiological significance which might be attached to the presence of the pigment. One can only suggest that like the haemoglobin the luteo-albumin may be of importance in connection with the oxygen supply of the body. This is not probable. The ease with which the carotin is increased and decreased in the blood serum as shown by the feeding experiments, seems to preclude the possibility of the carotin being absolutely essential to the life of the cow.

A STUDY OF THE HIGH COLOR OF COLOSTRUM MILK FAT.

Considerable data was given in the preceding Bulletin of this series, which showed that colostrum milk fat from all breeds of cows is characterized by a very high content of carotin. In view of the results obtained in the study of the pigment of the blood serum, it seemed very probable that this interesting phenomenon was due to a great accumulation of the carotin in the blood serum just previous to parturition. In order to obtain some definite experimental evidence in support of this supposition, blood was drawn from the jugular vein of a pure bred Jersey cow (No. 23), when she was dry and three days previous to parturition. The amount of color in 10 c.c. of the blood

1. After standing a short time under the alcohol.

was determined in the manner previously described. Thirty days after calving the blood was tested again. The color of the milk fat was determined at this time also. The color of the blood serum of another Jersey cow was determined at another time twelve hours previous to the time it was judged she would give birth to a calf. The data obtained in the two experiments are given in Table 7.

TABLE NO. 7.—RELATION OF BLOOD SERUM COLOR TO COLOR OF COLOSTRUM MILK FAT.

Remarks.	Color of serum		Color of fat.	
	Yellow	Red	Yellow	Red
Three days before parturition (Jersey Cow No. 23.)	42.0	0.8		
Thirty days after parturition (Jersey Cow No. 23.)	50.0	2.0	64.0	1.8
Twelve hours before parturition (Jersey Cow No. 2.)	29.0	0.2		

It is readily seen that another explanation must be sought for the high color of colostrum milk fat, other than an accumulation of carotin in the blood. No doubt a certain amount of storing up of carotin does occur if a cow is dry previous to parturition and the serum is low in color at the time of drying up, it being supposed of course that the food contains a plentiful supply of carotin. The data presented in Table 7, when coupled with the data in Tables 3 and 4, show very clearly that under normal conditions the amount of pigment carried by the serum does not exceed a certain maximum point that appears to be practically the same for all cows, regardless of breed. This is not abnormal when it is considered that the carotin of the blood serum is in combination with a protein which no doubt comprises a more or less constant proportion of the blood.

This result forces us to the same conclusion reached in connection with the study of the physiological relation between food, blood serum and milk fat carotin, namely that other factors, among which may be the composition of the milk, must be taken into consideration in explaining the pigmentation of milk fat. In the case of the high color of colostrum milk, some facts stand out that seem to have a special bearing upon the phenomenon. For instance it is a well-known fact that the milk drawn for the first few days after parturition has

a very abnormal composition which is characterized by a very low fat percentage and a very high protein content, the largest proportion of which is albumin. Unpublished data are at hand which show a composition of some colostrum milks of 1.3 per cent fat and over 4.5 per cent albumin. When it is considered that the carotin is carried by the blood in combination with an albumin, and when it is also taken into account that the source of the lactalbumin is undoubtedly, at least partially, the serum albumin, a most plausible explanation of the high color of colstrum milk fat is at once apparent. It is also apparent that this high color will continue until the milk has reached a normal composition or until the blood supply is depleted. That this will occur regardless of breed is also readily explained since data show that the maximum color of the blood serum does not materially differ with different breeds.

DISCUSSION OF RESULTS.

The results of the foregoing studies in regard to the yellow lipochrome of the blood serum of the cow do not require any extended discussion. Following the interesting discoveries set forth in the preceding papers in regard to the nature of the pigments of milk fat and body fat and their simple physiological relation to the carotin and xanthophylls of the food which the cow receives, it was not surprising to find that the hitherto practically unknown lipochrome of the blood serum of the same animal is also chiefly carotin and bears the same relation to the food as the milk fat carotin. We are thus able to establish the connecting link between the food carotin and the carotin of the milk fat, body fat carotin, corpus luteum, etc., of the cow.

One of the most important results of this study was the discovery that the carotin is not transmitted to the milk glands and body cell from the food by means of simple solution in the blood serum, but is on the other hand carried through the body in combination with an albumin of the serum.[1] This fact is undoubtedly of considerable importance in connection with the entire phenomenon of the pigmentation of the milk fat. It may be safely predicted that all the factors which surround this phenomenon are in some way dependent upon this fact, and all these factors will not be known until it is clearly understood what part this caroto- (or luteo-) albumin

1. Incidentally this discovery has resulted in the addition of a new chromoprotein to the list of conjugated proteins. This is itself of considerable physiological interest.

plays in the formation of the milk fat. The same holds true for the body fat.

The readily demonstrated fact that the withdrawal of carotin from the food results in a marked decrease in the color of the milk fat being secreted or in the body fat being formed, shows that the albumin which carries the carotin in the blood serum does play a definite part in the formation of both milk fat [1] and body fat and no doubt also in the formation of the corpus luteum.

The whole phenomenon offers many difficult and interesting problems for future study. Many of these when solved will undoubtedly throw light upon the chemistry of the mechanism of milk secretion.

SUMMARY.

1. The well-known lipochrome of the blood serum of the cow is, like the lipochrome of the milk fat, body fat, etc., of the same animal, composed principally of carotin, the widespread hydrocarbon pigment of plants. Associated in small quantity with the carotin of the serum, probably dissolved in the fat of the blood, are one or more xanthophyll pigments, which are always found in more or less variable quantities associated with the carotin of plants.

2. The carotin and xanthophylls of the blood serum are derived from the food and furnish the normal source for these pigments in the milk fat and body fat, etc. A variation in the quantity of these pigments in the food results in a corresponding variation in the amount found in the blood serum and milk fat. Body fat formed during this time will be also affected.

3. The carotin is carried by the blood serum in combination with an albumin. The combination is a very firm one. Lecithin and cholesterol are probably a part of the combination. We propose the name caroto-albumin for this new chromo-protein of the blood.

4. The caroto-albumin of the blood serum of the cow is probably of importance in the formation of the milk fat, body fat and corpus luteum of the cow. It is doubtful if this new pigmented protein is of importance in the oxygen respiration of the body.

5. The lactalbumin of cows' milk may, among other factors, be related to the color of the milk fat. There appears to be a special relation here in connection with the high color and the high albumin content of colostrum milk.

1. The presence of both cholesterol and lecithin in the caroto-albumin may explain the origin of these lipoids, as well as carotin in butter fat.

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B. CAROTIN AND XANTHOPHYLLS DURING DIGESTION

The establishment by us [1] of both a chemical and physiological relation between the carotin and xanthophylls of plants and the yellow lipochromes of the milk fat, body fat, blood serum and corpus luteum of the cow has shown that it is the carotin that is by far the more important in pigmentation of the animal body. It is a well-known fact that xanthophylls are as abundantly and sometimes more abundantly distributed in vegetable matter than carotin. The question naturally arises then, why carotin is the pigment which is principally taken up by the cow's body, and why the xanthophylls appear there only in very small quantity. This seemed to us to be an important physiological question.

It will readily be recognized that a question of this nature is not easily answered. It may therefore be stated in advance that the results of our studies were not as satisfactory as was anticipated. The data are presented, however, for what value they may possess, since opportunity was not presented for a further study of the question. The data are of some value, at least, in that a number of facts are presented which are sufficiently related to advance a fairly acceptable theory in regard to the question.

METHODS OF STUDY

Several methods of study which did not appear to offer many difficulties, seemed available, by which it was thought light could be thrown on the question. One method was to study the action of the various digestive fluids, both natural and artificial, on fresh crude residues of the amorphous carotin and xanthophylls of plants. Another method was to study the nature of the unsaponifiable pigment extracts at various places along the digestive tract of the cow. A third method was closely related to the second and consisted in a study of the unsaponifiable yellow pigments excreted under conditions where unassimilated or undestroyed carotin and xanthophylls of the food would be likely to appear unchanged in the feces. Lack of time made a thorough study of all the methods impossible so that only the significant features of the results of each study will be given.

1. Missouri Agricultural Experiment Station Research Bulletins Nos. 10 and 11, this Bulletin, p. 415 (1914); Jour. Biol. Chem. pp. 191, 211, 223 (1914).

THE ACTION OF DIGESTIVE JUICES

The following solutions were added to equal portions of carotin and xanthophylls [1] in test tubes, and the tubes plugged with cotton and set aside at 40°C. Observations for signs of decomposition were made every day for five days.

Tube 1. Five cc. of 0.25 per cent HCl solution of pepsin.

Tube 2. Five cc. of 0.25 per cent HCl solution of filtered gastric juice from the fourth stomach of a Jersey cow.

Tube 3. Five cc. of 0.25 per cent Na_2CO_3 solution of trypsin.

Tube 4. Five cc. of 0.25 per cent Na_2CO_3 solution of extract from pancreas of a Jersey cow.

Tube 5. Five cc. of 0.25 per cent Na_2CO_3 solution of trypsin plus 5 cc. of fresh bile from a Jersey cow.

Tube 6. Five cc. of 0.25 per cent NaCO solution of pancreatic extract [2] plus 5 cc. of fresh bile.

1. The carotin and xanthophylls were isolated as follows: 200 grams of air-dried, powdered, green alfalfa leaves were shaken with three litres of 10 per cent alcoholic petroleum ether for two days, and then with 1 litre of CS_2 until the solvent had taken up as much pigment as possible. The carotin and xanthophylls were isolated from each extract and combined. Each solution was now concentrated to 50 cc. and divided into ten parts. These were put into test tubes and the solvent driven off at a low temperature. The residues were used for the studies reported above.

The carotin and xanthophylls were isolated from the alcoholic petroleum ether extract as follows: The xanthophylls were removed from the extract by shaking with an equal volume of 80 per cent alcohol. The carotin in the petroleum ether was now freed from chlorophyll by shaking with an excess of $CaCO_3$, the solution was now evaporated into alcohol and transferred to ether by diluting with much water after the addition of ether. The solution was freed from traces of chlorophyll that had escaped absorption by the $CaCO_3$ by shaking with 30 per cent alcoholic potash. The ether was then freed from alkali with distilled water. This ether solution of carotin was combined with the similar solution obtained from the CS_2 extract as described below. The 80 per cent alcohol, containing the xanthophylls, was partially freed from chlorophyll by shaking with moist animal charcoal for one hour. The pigments were then transferred to ether, the remainder of the chlorophyll being removed by 30 per cent alcoholic potash as in the case of the carotin. The ether solution was then washed free from alkali and added to the xanthophylls obtained from the CS_2 extract as described below.

The carotin and xanthophylls were isolated from the CS_2 extract as follows: The extract was concentrated into 95 per cent alcohol and after filtering was saponified with KOH. The pigments were extracted from the soap with ether. The ether was washed free from alkali and evaporated into alcohol. The carotin and xanthophylls were separated by differentiation between petroleum ether (b. p. 30-50°C.) and the alcohol.

2. The pancreatic extract was prepared by extracting a freshly ground cow's pancreas with 150 cc. of 30 per cent alcohol for 24 hours, straining off the extract, filtering and neutralizing with KOH and 0.5 per cent Na_2CO_3. To prepare the above solution an equal volume of 0.5 per cent Na_2CO_3 was added.

Tube 7. Five cc. of neutral solution of pancreatin.

Tube 8. Five cc. of neutral pancreatic extract.

Tube 9. Five cc. of neutral pancreatic solution plus 5 cc. of fresh bile.

Tube 10. Five cc. of neutral pancreatic extract plus 5 cc. of bile.

The pepsin, trypsin and pancreatin were Merck's U. S. P. preparations.

A set of ten tubes was also prepared containing equal portions of the xanthophylls of yellow corn.[1]

The following results were obtained. *Carotin*: Bleaching occurred only in the tubes containing neutral and alkaline pancreatic extracts. In the same tubes plus bile there was no decoloration. The bile had no solvent action on the carotin, which was in marked contrast to the xanthophylls, as noted below. *Xanthophylls*: The pigments in tubes 1, 3 and 4 were largely decolorized at the end of the second day, while those in tubes 2, 7 and 8 retained their color after the fifth day. No observations could be made on the tubes containing bile until the fifth day on account of the fact that the bile had completely dissolved the pigments as soon as it was added. The pigments were examined by desiccating the contents of the tubes with plaster of Paris and extracting with ether. Marked bleaching had occurred in all the bile tubes. *Corn Xanthophylls*: There was marked destructive action of these pigments in all the tubes except those containing bile. The corn xanthophylls, like the xanthophylls from the alfalfa, were readily soluble in bile.

The most significant feature of the above results is the marked difference in the solubility of carotin and xanthophylls in bile, the surprising result being the very slight solubility of the carotin. This was confirmed quantitatively using carotin from another source and the bile from several different cows. The results are given in Table 1. The carotin used was a freshly prepared ether solution of carotin from the carrot. Equal volumes of this solution were evaporated at a low temperature and the residues treated with 10 cc. of bile from each of four cows. After standing for several days with frequent shaking the bile was filtered and 5 cc. of the filtrate desiccated with plaster of Paris. This was extracted with ether until colorless. The extract in each case was concentrated to a low volume, made up to 12.5 cc. with absolute alcohol, and the color of the solution measured in the Lovibond Tintometer.

1. This was the unsaponifiable pigment of the corn which was more soluble in 80 per cent alcohol than in petroleum ether.

TABLE NO. 1.—THE SOLUBILITY OF CAROTIN IN BILE.

Experiment No.	Source of bile	Carotin used		Carotin in bile		Blank*	
		Yellow	Red	Yellow	Red	Yellow	Red
1.	Jersey	57.0	2.0	3.0	0.6	1.0	0.2
2.	Angus	57.0	2.0	9.0	0.8	1.0	0.2
3.	Holstein	57.0	2.0	10.0	0.9	1.0	0.2
4.	Holstein	57.0	2.0	10.5	1.0	1.0	0.2

*The blank is the amount of color extracted from 5 c.c. of bile alone, after desiccation with plaster of Paris.

An interesting feature in the above table is the apparent greater solubility of carotin in the bile of Holstein cows, than in the bile of Jersey cows. If this is confirmed by future study, considerable significance could be attached to it in explaining, at least partly, the differences between the two breeds in the amount of carotin that is secreted in the milk fat.

CHARACTER OF THE PIGMENTS ALONG THE DIGESTIVE TRACT

The plan in this part of the study was to examine the pigments which could be extracted from the material at various places along the digestive tract of several cows. Material was obtained from one Holstein cow and two Jersey cows at slaughtering, from each of the three stomachs just before the food entered the next part of the digestive tract, from three places in the small intestines, from the caecum, and from the large intestine. One or two hundred grams of material were either dried on the steam bath or desiccated with plaster of Paris, and the resulting mass in either case extracted with CS_2. The solubility, spectroscopic, and adsorption properties of the extracted pigments were carefully noted. The pigments were thus differentiated into carotin and xanthophyll constituents as well as classified as belonging to either of the two groups.

The results of the study were not satisfactory, in that there was no uniformity among the several cows in regard to the character of the pigments found at any particular place, although all the animals were receiving a ration which should have furnished an excess of both carotin and xanthophylls. The reason for this is not obvious. It might be thought that the partial drying in some cases destroyed the pigments. Possibly this occurred to some extent, but it would not

account for the lack of uniformity where this method of desiccation was not employed.

No further discussion will be given this study. Mention has been made of it merely because the method seems to be a valuable one, and will warrant further application.

THE EXCRETED PIGMENTS

For this study the feces of a cow were examined, in a feeding experiment where the carotin and xanthophylls were furnished by the feeding of carrots only. The balance of the ration was composed of grain and timothy hay almost free from carotin and xanthophylls.

The method of demonstrating the character of the pigments in the feces was to desiccate a quantity of fresh feces with plaster of Paris and extract the mass with pure carbon bisulphide. The extract was concentrated and studied spectroscopically, and also by means of a Tswett chromotogramm. The relative solubility properties of the pigments thus found were studied, and also the spectroscopic properties of the pigments thus separated.

In this way it was found that when the cow was receiving 50 pounds of carrots per day, both carotin and xanthophylls were abundantly present in the feces. This continued for six days after the carrots were withdrawn from the ration, although it was possible to detect but little xanthophyll during this time.

DISCUSSION OF RESULTS

Combining the results of the above experiments, the appearance of carotin in the cow's system when fed in excess may be explained on the ground of its greater stability toward the digestive processes, as shown by the digestion experiments, and the abundant appearance of the pigment in the feces. The failure of the xanthophylls to appear to any extent in the cow's system may be due similarly to the fact that they are apparently more easily destroyed [1] during digestion. Some of them that escape destruction are undoubtedly taken up by the bile and thus enter the system through the portal circulation. Some oxidation probably takes place in the liver. If fat is present to any extent some of the xanthophylls will evidently be taken up and

1. Willstätter and Mieg. (Ann. d. Chem. 355, p. 1, 1907), state that xanthophylls are very sensitive toward acids. This would lead one to expect that they would be largely destroyed by the gastric juice. Our results were contradictory in this respect. We found an artificial gastric juice to destroy the xanthophylls but the natural gastric juice from the fourth stomach of a cow apparently had no effect on them.

enter the blood dissolved in fat. In this connection it is of interest to recall that we have shown [1] that there is evidence to indicate that what xanthophylls can be found in the blood are present dissolved in fat.

An additional possible explanation of this whole question should not be overlooked, however, namely, that the difference in the proportion of carotin and xanthophylls taken up by the cow's body may be due entirely to the difference in chemical composition between carotin and xanthophylls. Carotin is an unsaturated hydrocarbon and is furthermore capable of combining with a protein of the blood, as we have shown.[2] The xanthophylls, on the other hand, are carbon, hydrogen and oxygen compounds, in fact are chemically carotin-dioxides. Although still unsaturated bodies, their slight difference in composition from carotin, may prevent their combination with the serum albumin, thus making it impossible for them to appear to any extent in the blood and fatty formations of the cow's body. If fat played a greater part in the food of the cow, the xanthophylls would undoubtedly appear to a greater extent in the body of this animal.

SUMMARY

1. Carotin is assimilated from the food of the cow in preference to xanthophylls partly because of its greater stability toward the juices of the digestive tract. Xanthophylls are much more soluble in bile than carotin,[3] which probably accounts for their appearance in the fat of the blood.

2. It is probable that carotin forms by far the greater part of the lipochromes of the cow's body chiefly on account of its ability to form a compound with one of the proteins of the blood. The xanthophylls, being of different composition, probably are not capable of forming such a compound.

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1. This Bulletin, page 422; Jour. Biol. Chem. 17, p. 211, 1914.
2. *Ibid.*
3. A confirmation of the very slight solubility of carotin in bile is seen in the recent finding of Fischer and Röse (Zeit. f. Physiol. Chem. 88, p. 331, 1913), that the gall stones of cows contain crystallizable carotin. No xanthophylls were found.

C. THE PIGMENTS OF HUMAN MILK FAT

The discovery of the chemical and physiological relations of the pigments of the fat of cow's milk to the carotin and xanthophylls of plants naturally opens the question whether the pigments which characterize the fat of other animals are of a similar character. Opportunity was not afforded to study this question with any domestic animals other than the cow. An opportunity, however, was offered to investigate the character of the pigments which sometimes give a high color to the fat of human milk.

The methods used for studying the character of the pigments were the microscopic ones used in the preceding studies. The adsorption properties were not studied, however, the demonstration being confined to the observation of the absorption bands and the relative solubility properties.

The fat from two samples of human milk from different sources was used. Very little was known in regard to one of the samples, it having been sent to the laboratory for analysis by a well-known physician of the community. The other sample was taken by one of us from a woman who had just given birth to a child, and represented a portion of the milk of each day of the first few days of lactation. Some further observations in regard to this sample will be reported below.

Experiment No. 1

This was the sample in regard to which very little was known, with the exception that it was a bona fide sample of human milk. The milk had a faint yellow tint. The volume of milk used was approximately 125 c.cm. The milk contained about 3.5 per cent fat and therefore yielded a little over 4 grams of fat. The fat was obtained from the milk by precipitating it along with the proteins. To do this the milk was acidified with acetic acid, a pinch of salt added, and the milk brought to a boil. The precipitated proteins, when filtered off, had a bright yellow color, due to occluded fat. The fat was dissolved out with hot 95 per cent alcohol.

After concentrating the alcoholic extract, the fat was saponified by adding a small piece of KOH and boiling for about one hour. The pigment was readily extracted from the soap by ether, after dilution with water. The golden-yellow ether solution was washed with water and evaporated to dryness. The residue dissolved at once in carbon bisulphide with a red-orange color and in this solution

showed two beautiful absorption bands, and possibly a third. The CS_2 was carefully evaporated. A part of the residue which remained was difficultly soluble in absolute alcohol, but readily dissolved when a little petroleum ether was added. When differentiated between petroleum ether and 80 per cent alcohol the combined pigment was readily divided into two apparently equal proportions with perhaps slightly more color in the petroleum ether layer.

The pigment of the petroleum ether layer gave a red-orange carbon bisulphide solution showing two strong absorption bands and a third faint one, the measurements of which are given in Table No. 1 below.

TABLE NO. 1.—ABSORPTION BANDS OF CAROTIN AND XANTHOPHYLLS FROM HUMAN MILK FAT.

Experiment.	Measurements of absorption bands.	
	Carotin	Xanthophylls.
No. 1	I. 225—244 II. 262—280 III. 300—319	I. 234—253 II. 275—293 III. 320—
No. 2	I. 225—242 II. 265—282 III.	I. 232—252 II. 273—293 III. 312—330

The pigment of the alcoholic layer gave a yellow-orange, carbon bisulphide solution showing two good absorption bands and end absorption, the measurements of which are given in Table 1.

Experiment No. 2

As stated above, this sample of human milk was taken by one of us and represented the milk of the first few days of lactation including the colostrum milk. The milk itself was characterized by a high yellow color and the fat which rose to the top of the sample had a very deep yellow color. About 350 c.cm. of milk were obtained. The fat percentage being between 5 and 6, nearly 20 grams of fat were yielded for the study of the pigments.

The fat was obtained from this sample of milk in a manner very similar to that used in the preceding experiment. The proteins and fat were precipitated together by adding a little salt and also con-

siderable ammonium sulphate, acidifying with acetic acid and bringing to a boil. The precipitate was filtered off on a Büchner funnel. The layer of protein and fat had a golden-yellow color. The fat was extracted with hot alcohol and ether. The golden-colored extract was evaporated to dryness and the fat dissolved away with ether. Alcohol was added and also 5 grams of KOH and saponification of the fat allowed to proceed on the steam bath for one-half hour. The pigment was extracted from the diluted soap with ether. After thorough washing with distilled water, the ether was evaporated carefully to dryness. The residue had a deep red color. It dissolved at once in petroleum ether (b.p. 30°-50°C.).

The pigment in this solution was now differentiated between the petroleum ether and 80 per cent alcohol. In this way it was divided into two portions which were about equal as far as could be detected by the color of the two solutions, with perhaps slightly more color in the 80 per cent alcohol.

The pigment in the petroleum ether layer gave a blood-red colored carbon-bisulphide solution which showed two absorption bands and considerable end absorption. The measurements of these bands are given in Table 1.

The pigment in the 80 per cent alcohol layer gave an orange-colored carbon-bisulphide solution which showed three distinct absorption bands. The measurements of these bands are given in Table No. 1.

DISCUSSION OF RESULTS

The results of the above experiments show very clearly that the fat of human milk may be tinted with the same pigments found in the fat of cow's milk. The relative proportion of carotin and xanthophylls in human milk fat is much more nearly equal than in the fat of cow's milk. This is not surprising when it is considered that there is strong evidence that the xanthophylls are conveyed through the body dissolved in fat, and when it is also considered that fat plays a much greater part in human food than in the food of the cow.

An especially interesting fact brought out by these brief studies is that colostrum milk fat of the human is characterized by a very high color just as is the case with the fat of the colostrum milk from cows. In the experiment here reported, one of us had occasion to observe that after about ten days the milk fat from the same woman was very much lighter in color than during the first few days of lactation. The milk was also observed at intervals for a period of

several months. Considerable variation in the color of the fat was noticed. Although it was not possible to accurately trace the cause of this variation, as we did in the case of cows in an earlier paper of this series, it was undoubtedly due to changes in diet.

In conclusion it may be stated that all students of human anatomy are familiar with the fact that the fat on the human body is often characterized by a marked yellow color. In view of the fact that the pigments of the milk fat and body fat of the cow are identical, it must therefore be concluded that the pigments of the milk fat and body fat of humans are identical.

SUMMARY*

1. The fat of human milk may be tinted by carotin and xanthophylls, the pigments which characterize the fat of cows' milk. The relative proportion of carotin to xanthophyll in human milk fat is much more nearly equal than in the fat of cows' milk.

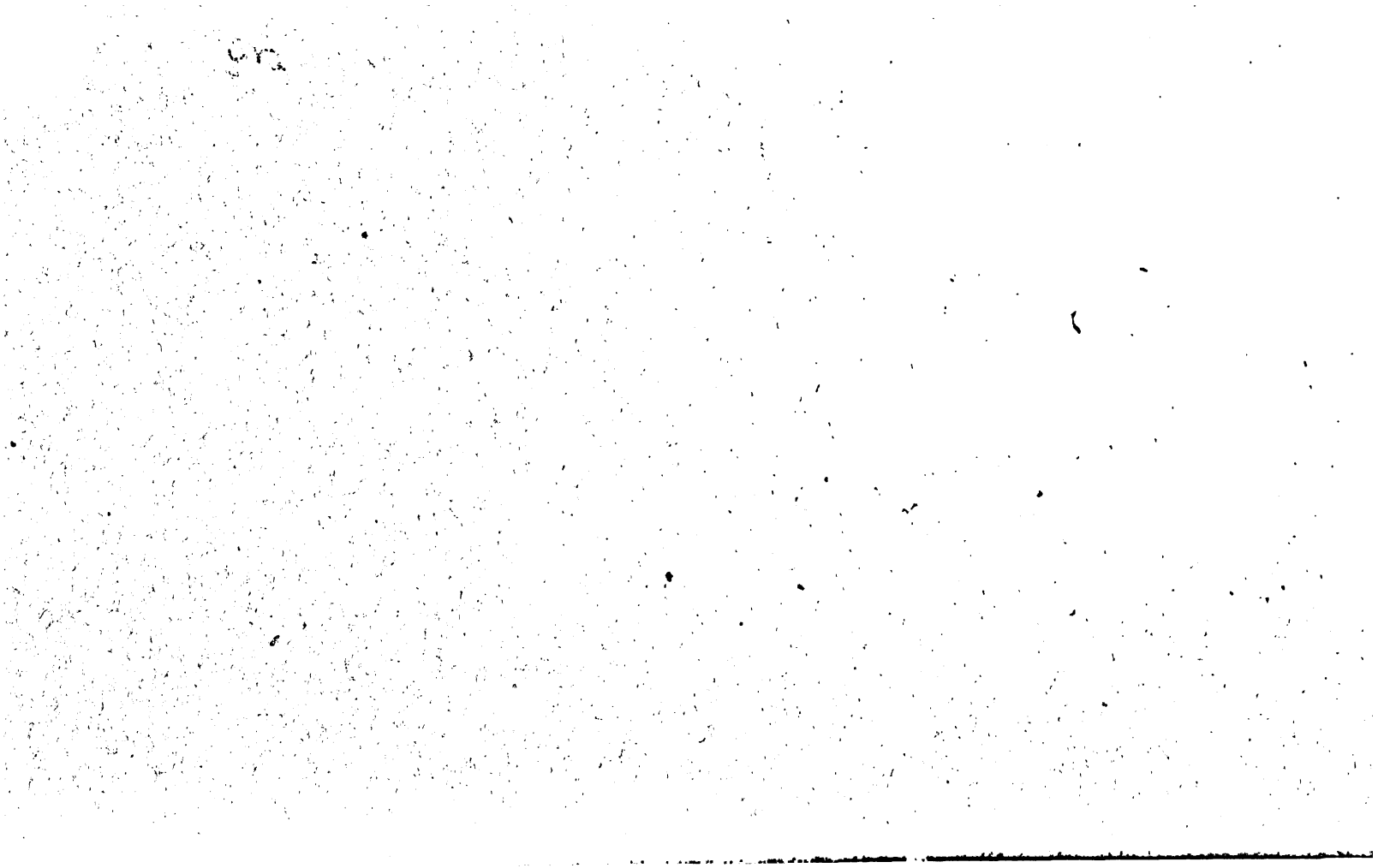
2. The colostrum fat of human milk is characterized by a very high color as is the case with the fat of the colostrum milk of cows.

3. The pigment of human body fat is no doubt identical with the pigment of human milk fat.

*See page 438 for summary of "The Yellow Pigment of Blood Serum."
See page 446 for summary of "Carotin and Xanthophylls During Digestion."

BIOGRAPHY

Leroy Sheldon Palmer was born in Rushville, Illinois, on March 23, 1887. He received his common school education in the public schools of the city of St. Louis, Missouri, graduating from the Central High School of that city in June, 1905. He entered the School of Engineering of the University of Missouri in September, 1905, and received the degree of B.S. in Chemical Engineering in June, 1909. During the summer of 1909 he was Chemical Assistant for the United States Bureau of Fisheries, the work being conducted under the direction of Dr. Chas. W. Greene at Columbia, Missouri. He was appointed Fellow in Chemistry at the University of Missouri for the year 1909-1910, but resigned in October, 1909, to become Assistant Chemist in the Co-operative Government Dairy Research Laboratory of the University of Missouri, being appointed by the Dairy Division of the Bureau of Animal Industry of the United States Department of Agriculture. He pursued graduate work in the University of Missouri during the years 1909-1910 and 1910-11, and received the degree of M.A. in June, 1911. He was appointed by the Dairy Division of the United States Department of Agriculture in October, 1911, as the Government Representative in the Co-operative Dairy Research Laboratory of the University of Missouri. He pursued work in the Graduate School of the University of Missouri during 1911-1912 and 1912-13. He was appointed Assistant Professor of Dairy Chemistry and Assistant Chemist to the Experiment Station in the Department of Agricultural Chemistry by the University of Missouri in April, 1913.



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